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Experimental Manipulation of Sexual Antagonism in *Drosophila melanogaster*

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A thesis submitted to the University of Sussex
for the degree of Doctor of Philosophy in Biological Sciences

January 2017

Declaration

I certify that all materials in this thesis that are not my own work have been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature.....

Katrine Koch Lund-Hansen

January 2017

Abstract

Despite the benefits of sexual reproduction, sharing a genome can put constraints on the evolvability of a species. This is due to sexual conflict, where the interests of each sex is in direct opposition to one another, and the benefit of one sex can be the cost of the other sex (i.e. sexual antagonism). Sex chromosomes have been the focus of much of the research done on sexual conflict due to their unique nature and are particularly interesting in the context of sexually antagonistic variance.

In the first experiment (**Chapter 2**), I used experimental evolution to investigate the standing sexually antagonistic variation on the X-chromosome of the common vinegar fly, *Drosophila melanogaster*. Unlike most other experimental evolution experiments where selection has been limited to males, I limited the inheritance of the X-chromosome to females only. I used a non-recombining X-chromosome balancer to control the inheritance of the female-limited X-chromosome. Throughout the evolution experiment, I tested different phenotypic traits that have previously been shown to be sexual antagonistic, as well as investigating how the transcriptome changed through female-limited selection (**Chapter 3**). The results were mixed but indicated that limiting selection of the X-chromosome to females could, to some extent, change the antagonistic variation and move traits towards the female optimum. In the second experiment (**Chapter 4**), I exchanged sex chromosomes between populations with divergent geographic origins. I used flies with special genetic constructs (e.g. autosomal balancers, fused-X chromosomes) to control the population crosses, so that sex chromosomes were introduced into a new background without any prior interaction. I found that introducing a novel sex chromosome increased male reproductive fitness through improved sperm competition at the cost of offspring viability. 25 generations after introducing the novel sex chromosome (**Chapter 5**), the increase in male fitness was undetectable and their fitness was again the same as the wild types. Collectively, this indicates an antagonistic coevolution between the sex chromosomes.

Together, these two experiments shed new light on sexual conflict and the antagonistic coevolution between the sexes at the genetic level, both between and within the sex chromosomes. These novel insights could help further the understanding of how sex chromosomes may affect speciation.

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Thesis Contents

Declaration.....	2
Abstract.....	3
Acknowledgements	4
Thesis Contents	6
List of Figures and Tables.....	8
Author's declarations	11
Chapter 1: General Introduction	12
Sexual conflict	12
Sexually antagonistic variance.....	12
The X-chromosome	13
Study species: <i>Drosophila melanogaster</i>	14
Experimental design.....	15
Chapter 2: Female-limited X-chromosome evolution in <i>Drosophila melanogaster</i>	
.....	18
Abstract.....	18
Introduction.....	18
Methods.....	20
Results.....	26
Discussion	28
Chapter 3: Gene expression of the female-limited X-chromosome in <i>Drosophila melanogaster</i>	39
Abstract.....	39
Introduction.....	39
Methods.....	40
Results.....	43
Discussion	44

Chapter 4: Sexually antagonistic coevolution between the sex chromosomes of <i>Drosophila melanogaster</i>	53
Abstract	53
Introduction	53
Methods	56
Results	63
Discussion	65
 Chapter 5: Experimental evolution of novel pairs of sex chromosomes in <i>Drosophila melanogaster</i>	 82
Abstract	82
Introduction	82
Methods	84
Results	86
 Chapter 6: General discussion	 94
Interlocus sexual conflict	94
Intralocus sexual conflict	96
Conclusion	97
 References	 99

List of Figures and Tables

Chapter 2

Figure 2.1: Protocol for the Female-limited X-chromosome evolution experiment ..	33
Figure 2.2: Protocol for assay crosses	33
Figure 2.3: Standardised fitness for the three treatment groups at generation 18	34
Figure 2.4: Standardised fitness for the three treatment groups at generation 40	34
Figure 2.5: Yeast stress assay for the five female genotype treatments	35
Figure 2.6: Thorax size in mm for the three treatment groups at generation 18	35
Figure 2.7: Thorax size in mm for the three treatment groups at generation 72	36
Figure 2.8: Growth rate for the three treatment groups	36
Figure 2.9: The percentage area of dark pigmentation on the last three abdominal tergites for the five female genotype treatments.....	37
Figure 2.10: Locomotion activity for the three treatment groups.....	37
 Table 2.1: Summary of results from ANOVA analysis of Linear Mixed-Effects Models.....	38

Chapter 3

Figure 3.1: Protocol for the Female-limited X-chromosome evolution	47
Figure 3.2: Protocol for assay crosses	47
Figure 3.3: Heat map of the 212 significant genes	48
Figure 3.4: The proportion of sex biased genes.....	49
Figure 3.5: Log2 fold change.....	49
Figure 3.6: Linear Discriminant Analysis.....	50
Figure 3.7: Diagram of the post hoc tests between the five different female genotype treatments.....	50
Figure 3.8: Venn diagram of the post hoc tests between the three male genotype treatments.....	51
 Table 3.1: GO term analysis of genes significant for the post hoc tests between FLXho/he females and Cwt/CFMho/he female treatments	51

Table 3.2: GO term analysis of genes significant for the post hoc tests between FLX males and Cwt/CFM male treatments.....	52
Table 3.3: List of significant genes in the model that were classified as sexually antagonistic	52

Chapter 4

Figure 4.1: Experimental setup of the round-robin cross	70
Figure 4.2: Protocol for crosses to create novel genotypes	70
Figure 4.3: Relative male fitness for 20 novel genotypes and five wild-type populations.....	71
Figure 4.4: Relative male fitness for wild-type and novel genotype treatments	72
Figure 4.5: Protocol for crosses to create novel sex chromosome genotypes	72
Figure 4.6: Relative male fitness for wild-type and novel sex chromosome genotype treatments.....	73
Figure 4.7: Change in relative fitness between novel genotypes and their wild-type populations.....	74
Figure 4.8: Thorax length in mm for wild-type and novel genotype treatments	75
Figure 4.9: Male longevity in wild-type and novel genotype treatments	75
Figure 4.10: Rate of courtship for wild-type and novel genotype treatments	76
Figure 4.11: Sperm defence (P1)	76
Figure 4.12: Sperm offence (P2).....	77
Figure 4.13: Male effect on relative female fecundity for wild-type and novel genotype treatments	77
Figure 4.14: Female longevity with male harassment for wild-type and novel genotype treatments	78
Figure 4.15: Total number of offspring from the male reproductive fitness assay. ...	78
Figure 4.16: Egg-to-adult viability for wild-type and novel genotype treatments	79
Figure 4.17: The proportion of male offspring for wild-type and novel genotype treatments.....	79
Table 4.1: Results of ANOVA analysis of Generalized Linear Models and Linear Mixed-Effects Models with block as random factors.	80

Table 4.2: The change in relative fitness with confidence intervals (CI) calculated by bootstrap for each of the novel genotype populations	81
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Chapter 5

Figure 5.1: Protocol for the experimental evolution of novel X/Y chromosomes	89
Figure 5.2: Relative male fitness for the eight evolved genotypes and the four wild-type populations	89
Figure 5.3: Relative male fitness for wild-type and evolved genotype treatments.....	90
Figure 5.4: Change in relative fitness between novel genotypes and their wild-type populations.....	90
Figure 5.5: Comparison of Δ Fitness at the start and end of the evolution experiment	91
Table 5.1: The eight genotypes chosen for the evolution experiment.	92
Table 5.2: Results of ANOVA analysis of linier mixed model with block as a random factor	92
Table 5.3: The change in relative fitness between the novel evolved genotypes and the wild-type background at generation 0 and 25	93

Author's declarations

Katrine Koch Lund-Hansen (KLH) wrote all chapters presented in this thesis with comments from Edward H. Morrow and Jessica K. Abbott. Further contributors for each chapter are detailed below.

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Chapter 5

The experimental evolution was performed by KLH. Data collection and statistical analyse by KLH.

Chapter 1: General Introduction

Sexual conflict

The fundamental definition of sex, that females have few large gametes while males have many small, is also what drives conflict between the two sexes. As the energy cost per gamete is higher in females than males, each egg is more valuable than a sperm cell; meaning females are less willing to waste an egg on a non-viable mating. Males can produce a larger number of gametes at the same energy cost, so they do not need to be equally concerned about non-viable matings and are therefore willing to mate more often (Bateman, 1948), though there is a limit to sperm production and the number of ejaculations (Dewsbury, 1982). This creates sexual conflict as males will try to increase their mating rate even at the cost to females, while females will try to reject unwanted mating, thus creating conflict between the evolutionary interests of the two sexes (Parker, 1979).

Sexual conflict can be divided into two categories, inter- and intralocus sexual conflict. Interlocus sexual conflict can be driven by any interaction between males and females, such as mating rates, fertilisation, parental investment, and female remating behaviour, and is defined as the conflict between a given locus in one sex and a different locus in the other sex (Chapman et al., 2003; Arnqvist and Rowe, 2013). Interlocus sexual conflict has been predicted to drive sexually antagonistic coevolution; an adaptation to increase fitness in one sex leads to counter-adaption in the other sex, potentially resulting in a perpetual cycle of adaptation and counter-adaptation (Perry and Rowe, 2015). On the other hand, intralocus sexual conflict occurs because the two sexes share the same genome but may have different phenotypic trait optima, and is defined as the conflict between alleles within a locus. If an allele is sexually antagonistic it is beneficial in one sex but harmful in the other sex (Bonduriansky and Chenoweth, 2009).

Sexually antagonistic variance

When the fitness optimum for a trait is not the optimal trait expression for either sex, the trait can be defined as a sexually antagonistic trait. This displacement from the optimum is also known as the gender-load and results in lower population fitness (Rice, 1992). To reduce the gender-load in females, sexually antagonistic selection

will act to move them closer to their optimum, while pulling males away from their optimum, thereby increasing the gender-load for males, resulting in decreased fitness. Sexually antagonistic selection in males will then respond to attempt to increase fitness again, which is why sexually antagonistic selection has been likened to a tug-of-war over trait expression. The evolution of sexual dimorphism has been suggested as a solution to this tug-of-war (Bonduriansky and Chenoweth, 2009; Cox and Calsbeek, 2009).

Sexually antagonistic variation has been shown in red deer, *Cervus elaphus* (Foerster et al., 2007), the vinegar fly, *D. melanogaster* (Rice, 1996, 1998; Chippindale et al., 2001; Rand et al., 2001; Pischedda and Chippindale, 2006; Long and Rice, 2007; Prasad et al., 2007; Morrow et al., 2008), the collared flycatcher, *Ficedula albicollis* (Merilä et al., 1997; Merila et al., 1998), zebra finches, *Taeniopygia gutta* (Price and Burley, 1993, 1994) and the ground cricket, *Allonemobius socius* (Fedorka and Mousseau, 2004) and is estimated to be widespread in wild populations (Cox and Calsbeek, 2009). The presence of sexually antagonistic variance in the genome and its effect on fitness have also been shown through sex-limited evolution experiments in *D. melanogaster* (Rice, 1996, 1998; Prasad et al., 2007; Abbott et al., 2013), where they found that removing selection on one sex changed trait expression in the other sex thereby allowing an increase in sex-specific fitness. They also showed that it was due to intralocus sexual conflict, as the non-selected sex had decreased fitness when expressing the selected genome.

The X-chromosome

Sex chromosomes have unique qualities that distinguish them from the rest of the genome, such as sex determination and sex-limited gene expression (Mank, 2012). These are some of the characteristics, which has led to the theory that sex chromosomes contribute to resolution of intralocus sexual conflict (Rice, 1984; Rice and Chippindale, 2002).

Genetic sex determination has evolved independently multiple times through evolutionary time, but the most widespread sex chromosome system is male heterogamety, XY (Charlesworth, 1991; The Tree of Sex Consortium, 2014). As there is no common origin of sex chromosomes, sex determination also varies between taxa. For example, the sex of most mammals is determined by a gene on the Y-

chromosome (Waters et al., 2007), but in *Drosophila* sex is determined by the ratio of X-chromosomes to autosomes (Bridges, 1925). There are, however, some common features between the systems, such as no recombination between X and Y, a degeneration of gene content on the Y-chromosomes, and a subsequent dosage compensation of X-linked genes (Charlesworth, 1996). Unlike the Y-chromosome, which is entirely male-limited, the X-chromosome spends time in both females and males, exposing it to different selection pressures. Due to the hemizyosity of the X-chromosome in males, there is a strong purifying selection of any X-linked male-lethal alleles whether dominant or recessive in females. In females, recessive X-linked female-lethal alleles can persist in a population if the non-lethal allele shows near or complete dominance, thereby masking the recessive allele from selection (Rice, 1984; Charlesworth et al., 1987). Following population genetics, two-thirds of all X-chromosomes are found in females at any point, so it might be expected that there is greater positive selection in females than males. This could result in ‘feminisation’ of the X-chromosome, which has been shown in *Drosophila* (Parisi et al., 2003).

In 1984, Rice proposed a theory for the preferential accumulation of sexually antagonistic genes on the X-chromosome (Rice, 1984). Sexually antagonistic male-beneficial alleles can increase in frequency even if they are female-lethal when homozygotic if they are X-linked recessive. X-linked dominant sexually antagonistic alleles are able to increase in frequency if they are female-beneficial. To empirically test this theory Gibson *et al.* (2002) created 20 iso-X lines and found that the *D. melanogaster* X-chromosome was enriched for sexually antagonistic variance. Further support was provided by Innocenti and Morrow (2010) who found an enrichment of transcripts having with sexually antagonistic relationships with fitness on the X-chromosome of *D. melanogaster*.

Study species: *Drosophila melanogaster*

The common vinegar fly, *Drosophila melanogaster*, from the large family Drosophilidae, has been used as a genetic laboratory model organism for over a 100 years since T. H. Morgan found a white eyed male in his populations (Morgan, 1910). Today, *D. melanogaster* are an important part of biomedical research and are used in many different fields to understand the complex biological processes, including genetics, developmental biology, and neurobiology (Jennings, 2011). After the whole

genome was sequenced in 2000 (Adams et al., 2000) *D. melanogaster* is now also used as a model for human diseases; 77% of human disease genes have a homolog in *D. melanogaster* (Reiter et al., 2001).

One of the reasons why *D. melanogaster* has become such a popular model organism is because it is easy to keep and relatively cheap to maintain in large populations. At a constant temperature of 25°C it takes a fly about 9-10 days to develop from egg to adult (Greenspan, 2004), so within a year a population of *D. melanogaster* can go through 25 generations, which therefore also makes it a good candidate for long term selection experiments – a strategy widely used within evolutionary biology. Another benefit is the vast range of genetic tools available to *Drosophila* scientists, such as numerous transgenic lines and balancer chromosomes for the three major chromosomes (X, 2, and 3, Ashburner et al., 2005), which makes it possible to have complete control over the genomic composition of offspring.

D. melanogaster is a sexually dimorphic species, with males being smaller than females and completely melanised on their last three abdominal segments. Males also have sex combs on their front legs, which they use for courtship, and external genitals at the end of the abdomen (Demerec, 1965). These are all good visual cues and make it easy to identify and sort flies by sex.

D. melanogaster is a male heterogametic species (XY), but genes on the Y-chromosome do not determine sex. Instead sex is determined by the ratio of X-chromosomes to autosomes, with a ratio of 2:2 producing a female even if it carries a Y-chromosome (Bridges, 1925).

Experimental design

Since Parker defined sexual conflict in the seventies it has been a rapidly growing field within evolutionary biology, and many aspects of sexual conflict have been tested both theoretically and empirically. The aim of this thesis is to further add to the empirical data of both inter- and intralocus sexual conflict. In **Chapters 2 and 3**, I did a female-limited X-chromosome evolution experiment to examine intralocus sexual conflict. I investigate how sexually antagonistic variance on the X-chromosome is relevant for female fitness (**Chapter 2**) and how gene expression changes under female-limited X-chromosome evolution (**Chapter 3**). In **Chapters 4 and 5**, I use population crosses to test coevolution between the sex chromosomes (X and Y) and

whether this interlocus sexual conflict is antagonistic in nature. For all experiments the major proportion of the experimental work was creating the fly populations that the subsequent phenotypic and transcriptome experiments were performed on.

Experimental evolution

Experimental evolution is a useful experimental tool to observe evolutionary processes in real time (Harshman and Hoffmann, 2000; Burke and Rose, 2009). Because experimental evolution occurs in a controlled environment with multiple replications of treatment and control populations, it is possible to disentangle the evolutionary response to selection from environmental effects on phenotypes (Harshman and Hoffmann, 2000). It can also happen quickly; many studies have shown a response to experimental evolution after 10-20 generations (Kawecki et al., 2012). It should be noted though, that the aspects of experimental evolution that make it a useful tool are also the same ones that can make it problematic to extrapolate to populations in the field. The laboratory provides a stable abiotic environment, with small fluctuations in temperature and humidity, abundant food, and no predators. The long-term directional selection enforced by experimental evolution is also different from natural populations where natural selection is not stable but can change rapidly (Harshman and Hoffmann, 2000; Burke and Rose, 2009). But, as a proof-of-concept to test a range of evolutionary theories experimental evolution, it can be helpful and unexpected results can be interpreted in a clear theoretical framework (Houle et al., 2003; Kawecki et al., 2012). Because *D. melanogaster* has a short generation time, and is easy to keep in large populations, it is a good candidate for experimental evolution (Burke and Rose, 2009).

Population crosses

Population crosses are a useful tool to investigate the role of sexual conflict in speciation. It is predicted that sexual conflict can lead to fast divergence between populations through sexually antagonistic coevolution, as traits for reproductive isolation are constantly changing at different rates (Gavrilets, 2000), and each population would have its own coevolutionary trajectory caused by random mutations and genetic drift (Futuyma, 2009). This hypothesis can be tested by crossing divergent populations, because females will have coevolved with, and developed resistance to, males from the same population, but are predicted to be harmed by

mating with males from an allopatric population (Chapman et al., 2003; Long et al., 2006). Even if the results from such population crosses are mixed, they still provide a relevant support for sexual conflict (Chapman et al., 2003).

Fly populations

As the base for all of my experiments, I used the outbred wild type population LH_M, which has been in the laboratory since 1991. It has been maintained at a steady population of 1792 breeding adults with non-overlapping generations for over 500 generations. Limiting the amount of live yeast available and the number of breeding pairs to 16 in each vial creates a competitive environment for the adults. This is also the case for larval density which is maintained at 150-200 eggs in each vial (for more details see Rice et al., 2005).

In **Chapters 2** and **3** I also used an X-chromosome balancer stock, FM7a (B^l , sc^8 , v^{Of} , w^a , y^{31d}). This is a highly inbred fly stock, so prior to using them in my experiments I backcrossed the balancer into the LH_M population multiple times.

In **Chapters 4** and **5** I used clone-generator (CG) females (C(1)DX, y , f , T(2;3) $rdgC$ st in ri p^P bw^D) for the sex chromosome crosses. I also used four other wild-type populations, Dahomey, Innisfail, Odder, and Tasmania provided to me from other fly laboratories (provided by Ary Hoffmann, Mads Fristrup Schou, and Stuart Wigby).

Chapter 2: Female-limited X-chromosome evolution in *Drosophila melanogaster*

Abstract

Several studies have investigated sex-specific, sexually antagonistic constraints on attaining fitness optima; this has previously been investigated through a series of male-limited evolution experiments. In this chapter I employ a female-limited X-chromosome model, using an X-chromosomal balancer to limit inheritance of the X-chromosome to the matriline, and removing exposure to male selective constraints. Both approaches eliminate the effects of sexually antagonistic selection on the genome (or part of it), permitting evolution towards a single sex-specific optimum. After several generations of selection some, but not all, phenotypic traits measured here moved towards a female optimum. This may be due to the fact that the sexually antagonistic variance on the X-chromosome is not strong enough to impact all phenotypic traits or that the X-chromosome is already ‘feminised’ and therefore closer to a female optimum. This experiment adds to the growing list of evidence for sexually antagonistic variance in the genome and how this might constrain the evolution of sexual dimorphism

Introduction

While females and males differ in many biological aspects, such as behaviour, morphology, and physiology, they share, for the most part, the same genome. This can lead to evolutionary conflict, as each sex attempts to maximize its fitness, potentially to the detriment of the other sex (e.g. mating strategies, parental investment or seeking food resources). A shared genome cannot fully accommodate the interests of both sexes; there is, therefore, a theoretical expectation that this will limit each sex from reaching individual optima (Parker, 1979; Rice and Chippindale, 2002; Bonduriansky and Chenoweth, 2009). This is a particular problem when alleles that increase fitness in one sex are directly detrimental to the other, i.e. alleles may be sexually antagonistic. In cases where the two sexes have different phenotypic optima for a shared trait, alleles that affect this trait value will experience opposing forces of selection in the two sexes. Fixation of alleles favoured by selection in females leads to

counter-selection in males for alleles that move the trait value back towards the male optimum. This evolutionary process has led people to liken sexually antagonistic selection to a genomic tug-of-war between the sexes (Bonduriansky and Chenoweth, 2009).

Sexually antagonistic selection has been shown to be common in wild populations across many different vertebrate and invertebrate taxa (Cox and Calsbeek, 2009) and there is evidence that sexually antagonistic genetic variation is substantial within *Drosophila melanogaster* (Chippindale et al., 2001; Gibson et al., 2002). A simple and elegant way to test the presence of sexually antagonistic selection and its impact on shared phenotypic traits is sex-limited experimental evolution, where natural selection is limited to one sex. In theory, limiting selection to one sex frees traits from constraints imposed by intralocus sexual conflict, thereby allowing traits to evolve towards their phenotypic optimum, increasing sex-specific fitness. Similarly, if one sex is experimentally allowed to ‘win’ the evolutionary tug-of-war by applying a regime of sex-limited selection, the non-selected sex should have lower fitness and be further away from its ‘ideal’ phenotype when expressing the experimentally selected genome. Thus, limiting selection to one sex through experimental evolution allows the selected sex to gain fitness at the cost of the other sex, indicating that sexually antagonistic selection was present under ancestral conditions.

Previous evolution experiments have shown that it is possible to alter selection on the genome to favour one sex over another, thereby pushing trait values towards the optima of the selected sex. This was achieved either through genome-limited selection (Rice, 1996, 1998; Prasad et al., 2007; Abbott et al., 2013) or the removal or limitation of sexual selection (Holland and Rice, 1999; Pitnick et al., 2001; Nandy et al., 2013; Innocenti et al., 2014). So far, sex-limited evolution experiments have been restricted to allow selection in males only. It is therefore a natural extension to undertake a female-limited evolution experiment to test sexual conflict theory using a complementary design. By using an X-chromosome (chrX) balancer, which does not recombine with its homolog, it ensures a female-limited transmission of the chrX. I chose to limit selection to the chrX since both theory (Rice, 1984) and empirical evidence (Gibson et al., 2002; Innocenti and Morrow, 2010) indicates that the chrX is enriched for sexually antagonistic variance for fitness. Furthermore, I intended to explore any effects of sex-limitation using transcriptomic data (see Chapter 3), so I

anticipated that a design restricting sex-limitation to one genomic component would simplify results and interpretation.

In this chapter, I test the hypothesis that eliminating male selection on the chrX during a female-limited X-chromosome (FLX) evolution experiment would release sexually antagonistic standing genetic variation from the tug-of-war. As a result, I predict that expressing the experimentally feminised chrXs will shift trait values towards female-specific optima in both sexes. I also predict that female fitness would increase relative to experimental controls, to the detriment of male fitness. To estimate the effects of this evolution experiment, I tested a number of shared phenotypic traits previously shown to vary under male-limited (ML) evolution experiments: reproductive success/fitness, body size, and development time (Prasad et al., 2007). In addition, I tested locomotion activity, which has been shown to be a sexually antagonistic trait (Long and Rice, 2007), and abdominal pigmentation (females only). I subsequently examined gene expression profiles of the resulting genotypes from the FLX experiment and those results are discussed in Chapter 3. ML evolution experiments have shown that removing female selection resulted in increased male reproductive fitness to the detriment of female reproductive fitness, simultaneously decreasing the body size and an increasing development time (Prasad et al., 2007). I therefore predict that removing male-specific selection will produce the opposite result, increasing female reproductive fitness at the cost of male reproductive fitness, increasing body size, and decreasing development time. Long and Rice (2007) found a negative correlation between locomotion activity and female reproductive fitness, so females with higher locomotory activity had lower fitness. Thus, I expect that the FLX evolution experiment will result in decreased female locomotory activity, together with increased in female fitness.

Methods

Fly stocks

I used the LH_M stock as the base population for the evolution experiment. LH_M comes from a large laboratory-adapted population collected in central California in 1991 and founded from 400 inseminated females (Rice et al., 2005). This stock has been maintained in our lab for over 500 generations under the standard LH_M culturing protocol: at 25°C, 12-12 light-dark cycle, 60% relative humidity, and fed on

cornmeal-molasses-yeast medium (Rice et al., 2005). On day 12 after oviposition, 16 pairs of adult flies are randomly selected and placed in a food-vial with 6 mg yeast. The pairs are left in the vials for two days, and then flipped into fresh vials without yeast. The females can oviposit for 18 hours, after which all adult flies are discarded. The number of eggs is reduced to 150-200 per vial to maintain constant larval density.

To ensure female-limited selection of the chrX I used a chrX balancer (First Multiple; FM). FM functions as a normal chrX but does not recombine with the homologous chrX; it also carries phenotypic markers that enable simple tracking of balancer segregation. The FM7a balancer stock was obtained from Adam Chippindale, Queen's University. FM7a has the phenotypic dominant eye-marker Bar (B^1), as well as the recessive alleles for yellow body colour (y^{31d}), vermilion (v^{Of}) and white-apricot (w^a) eye colour, and extra bristles on the head (sc^8). In heterozygote flies, the B^1 presents as kidney-shaped eyes, making them easy to distinguish from wild-type and homozygotes flies (that have a narrow white bar-shaped eye). To introduce the FM7a balancer into the base population, I backcrossed the FM7a stock into LH_M for 12 generations, by crossing heterozygote (FM/X) females to LH_M males. To ensure that the mitochondrial genetic background was also derived from LH_M I crossed LH_M females to FM/Y males for one generation. Each cross was done with 672 breeding adults (♀16:16♂ per vial) in 42 vials.

After 12 generations of backcrossing, I established three treatment groups (see below), the experimental female-limited treatment and two controls, in four replicate populations per treatment, giving a total of 12 populations. Each treatment group was maintained as an adult breeding population of 448 individuals (♀16:16♂ per vial) in 14 vials with a minimum of 2,100 offspring.

Female-limited X-chromosome (FLX) treatment group – To ensure female-limited evolution of the chrX, heterozygote (FM/X) females were crossed to FM/Y males. Thus, females always inherited the evolving chrX from their mother and an FM balancer from their father (Figure 2.1). To have complete control over the female-limited inheritance pattern of the chrX, the FM/X females were collected as virgins every generation.

Control FM (CFM) treatment group – I used this treatment group to control for unforeseen effects of adaptation to the presence of the FM balancer in the FLX populations. To ensure that this control was closely represented the inheritance patterns of the chrX in a wild-type population, the chrX was present in males one-

third of the time. Every third generation FM/X females were cross to CFM X/Y males, so the chrX was passed from father to daughter (Figure 2.1). As with the FLX treatment group, all females were collected as virgins.

Control wild type (Cwt) treatment group – A group of wild-type flies was maintained under the same experimental conditions as the FLX treatment (virgin collection, smaller population size), but without sex-limited selection or the FM balancer. I was thereby able to control for the experimental protocol itself and for any effects that may be caused by a reduction in effective population size.

Recombination box – Because the chrX does not recombine with the FM balancer, the small effective population size of the chrX could lead to an accumulation of deleterious mutations due to genetic drift and/or hitchhiking (Futuyma, 2009). This finite population size could also slow selection on the experimental chrX due to the Hill-Robertson effect, where selection on one locus can affect the efficiency of selection on a second locus, especially if they are in linkage, thereby reducing the overall efficiency of selection (Hill and Robertson, 1966). Rice (1996) showed that allowing recombination in a small percentage of the experimental population could reduce these problems. To allow this, every generation 16 FM/X flies (4%) were removed from each of the evolving FLX populations to go through recombination. These 16 FM/X flies were crossed to X/Y males from the same populations, so the two chrXs could recombine in X/X females in the next generation. The X/X females were then crossed to FM/Y males, so the recombined chrXs can be returned to the evolving populations, as FM/X females. Using this method, one recombined chrX was added to each vial every generation, so 14 FM/X flies (3%) were returned to the evolving populations (see the recombination box for FLX in Figure 2.1). As the chrX in CFM is only co-located with the FM balancer in females, each generation the same number of flies are removed to undergo recombination as in the FLX treatment (see the recombination box for CFM in Figure 2.1).

Expression of evolved and control phenotypes

Because the FM balancer is a genetic tool used to control the female-limited selection, it is genetically irrelevant when I assay the flies and their phenotypes. Instead, I am interested in the phenotypic changes that are driven by the evolving chrXs. For all phenotypic assays I included two female genotypes: homozygote, females with two evolving chrXs, and heterozygote, females with one evolving and one wild-type chrX.

This presented a unique opportunity to investigate possible phenotypic differences between homozygote and heterozygote females. One generation before each assay I crossed FLX FM/X females to either FLX X/Y males (to produce FLX homozygote females) or Cwt males (to produce FLX heterozygote females; see Figure 2.2). I repeated this for CFM FM/X females, as their chrX is also inherited together with an FM balancer, mating them to either CFM X/Y males (to produce CFM homozygote females) or Cwt males (to produce CFM heterozygote females, see Figure 2.2). All males assayed carried either evolved FLX or CFM chrXs, giving in total of eight different genotypes per replicate population to test in each assay.

Female fecundity assay

Female fecundity was measured as the number of eggs laid by a female during an 18 hour period, which matches the period normally available to females from the base population (Rice et al., 2005). Five adult virgin females (collected on day 10 after oviposition) were combined with ten competitor virgin females and 15 males in a yeasted vial on day 12. The competitor females and males were from the outbred LH_M population homozygous for the visible brown eye (*bw*) genetic marker (LH_M-*bw*) that is recessive to the wild-type red eye-colour allele. After two days, the five target females were isolated in individual test tubes with food medium for 18 hours to oviposit. The females were then discarded, and the test tubes frozen so the eggs could be counted at a later date. This assay was performed at two time points, generation 15 and 41. At generation 15, the assay was performed in 10 replicates per female genotype per replicated population, thereby providing fitness estimates based on data from 200 individuals per genotype. At generation 41, the assay was performed in 12 replicates per female genotype per replicated population, thereby providing fitness estimates based on data from 240 individuals per genotype. Relative fecundity was calculated by dividing the fitness for each replicate by the maximum fitness across all replicates.

Female yeast stress level assay

This assay was performed following the same protocol as in female fecundity assay. When the five adult virgins were combined with the LH_M-*bw* flies, the vials had four different levels of yeast available 0mg, 5mg, 10mg, and 15mg. This assay was done in seven replicates per female genotype per yeast level per replicated population, giving

a fitness estimate for 140 individuals per yeast level per genotype. Relative fecundity was calculated by dividing the fitness for each replicate by the maximum fitness across all replicates. This assay was performed at generation 50.

Male fitness assay

Male fitness was measured as the proportion of live offspring sired by males carrying the target chrX. Five adult target males (day 13 after oviposition) were combined with ten competitor LH_M-bw males and 15 virgin LH_M-bw females in a yeasted vial. After two days, 14 females were isolated in individual tubes with food medium for 18 hours to oviposit. The females were then discarded, and the test tubes were left under standard LH_M conditions for 12 days. Adult offspring from each tube were counted and scored for eye-colour to assign paternity between target and competitor males. Since the wild-type (red) eye-colour allele is dominant to the bw allele, offspring with a red eye-colour can be assigned to the target males. As with the female assay, this assay was performed at two time points. At generation 18, the assay was performed in 10 replicates for each of the three males genotypes, thereby providing fitness estimates based on data from 200 individuals per genotype. At generation 39 and 40, the assay was performed in two blocks, with six replicates of the three male genotypes in each block, thereby providing fitness estimates based on data from 240 individuals per genotype. Relative fitness was calculated by dividing the fitness for each replicate by the maximum fitness across all replicates.

Thorax size

Body size was estimated using measurements of thorax length. On day 13 from oviposition, 20 flies from each genotype were placed in 95 % ethanol. Air-dried flies were measured using a Nikon SMZ800 dissecting microscope at 63x magnification fitted with an eyepiece graticule. The flies were measured lying on their right side, from the start of the prescutum to the end of the scutellum. This assay was done at generations 18 and 72.

Development time

Females (FLX and CFM: FM/X, Cwt: X/X) were collected as virgins on day 10 after oviposition and were cross to males (FLX and CFM: FM/Y or X/Y, Cwt: X/Y) on day 12. This ensured that the eclosing flies had the right genotypes for the assay, FLXho,

FLXhe, FLX male, CFMho, CFMhe, CFM male, and Cwt female and male. For each genotype and replicate, five vials were set up with 16 pairs in each. After the flies had interacted for two days, females were allowed to lay eggs in fresh vials for 18 hours. After discarding adult flies, the egg number was reduced to 150-200 eggs. On day nine after oviposition, vial observation commenced; they were subsequently observed approximately once every six hours until no flies had eclosed for seven consecutive observations. At every observation the vials were cleared of eclosed flies; these were scored for sex and phenotype. This assay was performed at generation 43.

Female abdominal pigmentation

30 females were placed in 95 % ethanol on day 12 after oviposition. Images of air-dried females were taken with a Nikon SMZ800 dissecting microscope at 63x magnification fitted with a Nikon Digital sight DS-Fi2 camera. Images were analysed in ImageJ 1.46r, using the *Area Fraction* measurement in the *Analyze* menu. *Area Fraction* measures the percentage of pixels in a selected area that have been highlighted in red using the *Threshold* tool. This gave an estimate of the percentage of dark pigmentation on the three terminal tergites of the abdomen. Flies for this assay were collected at generation 41.

Locomotion activity

On day 12 after oviposition, five adult non-virgin flies of the same sex and genotype were collected and placed in a vial. These vials were left for 24 hours to ensure that the flies had fully recovered from CO₂ anesthesia during collection. A large rectangle partitioned into eighths was drawn on each vial and one fly within one of eighths was randomly chosen for observation. The chosen fly was observed for 3s and it was noted if the fly was walking around (active) or not. Each vial was observed in 10 separate sessions on the same day. This assay was performed in five replicates per female and male genotype per replicated population in total 20 vials at generation 72.

Statistical procedures

All statistical analyses were conducted in R version 3.2.3 (R Core Team, 2015). As I wanted to measure the response to the evolution experiment in both sexes, I chose to include both in analyses where possible. For analyses that included both sexes, I used the Linear Mixed-Effects Model (*lmer*) command from the R package **lme4** (Bates et

al., 2015) with genotype (8 levels: FLXho, FLXhe, FLX male, CFMho, CFMhe, CFM male, Cwt female and male) and zygosity (3 levels: homozygous, heterozygous, or hemizygous for the chrX) as fixed factors and replicate population as random factor. Significance tests were obtained using the *anova* command. I standardised (mean = 0, standard deviation = 1) the raw fitness data to ensure male and female data were comparable. For the two analyses that included only female data (yeast stress level and pigmentation) I used *lmer* with female genotype as a fixed factor and replicate population as a random factor, adding a quadratic term for each. Since abdominal pigmentation is measured at the three tergites on each female, I also added female ID to account for repeated measurements in this model. For the locomotion activity assay, I tested the number of active and inactive flies against sex and genotype and zygosity using the Generalized Linear Mixed-Effects Models (*glmer*) command also from **lme4**, with family set as binomial. Replicated population was added as a random factor. Significance was tested with the *Anova* command from the *car* package (Fox and Weisberg, 2010). I tested for overdispersion in the model using the *dispersion_glmer* command in the **blmeco** package (Korner-Nievergelt et al., 2015).

Results

I decided to test the reproductive fitness at two time points (generation 18 and 40) during the FLX evolution experiment (Figure 2.1) so I could compare early adaption to long-term selection. I tested the reproductive fitness of five female genotypes and three male genotypes and compared their standardised fitness (Figure 2.2). I did not find any significant difference between the genotypes within nor between the different levels of zygosity at generation 18 ($P = 0.25$, see Figure 2.3 and Table 2.1) or generation 40 ($P = 0.09$, Figure 2.4 and Table 2.1).

Since female fecundity is correlated to the amount of live yeast available (Linder and Rice, 2005), I carried out a yeast stress level assay to test how capable the five female genotypes were at responding to varying availabilities of altering amount of available live yeast; the normal yeast dose of 6 mg may mask differences between the genotypes. I confirmed the results of Linder and Rice (2005) that there is a significant effect of live yeast availability on fitness ($P < 2e^{-16}$, Table 2.1). Furthermore, I found that the five female genotypes had significantly different responses to the different levels of yeast availability ($P < 2e^{-16}$, Figure 2.5).

Interestingly, the quadratic fitted curves for the two FLX genotypes had different trajectories, with FLX homozygotes having a lower fitness optimum at a higher yeast levels than FLX heterozygotes, indicating heterozygote advantage.

Both female fecundity (Robertson, 1957) and male fitness (Pitnick, 1991) have been shown to positively correlate to the body size in flies, so this correlation was tested by assaying body size at two time points during the evolution experiment. At generation 18, I found a significant difference in body size between all genotype treatments and zygosity ($P < 2.2e^{-16}$, Table 2.1). The FLX females were significantly larger than the other female genotype treatments, whereas the FLX males were only significantly larger than the Cwt males (Figure 2.6). At generation 72, there was also a significant difference in body size between the treatments ($P < 2.2e^{-16}$, Table 2.1), but neither FLX females nor males were still significantly larger than the other two treatments (Figure 2.7). This was largely driven by an increase in size of CFM females and males.

Body size has previously been shown to be positively correlated with developmental time (Chippindale et al., 2003), but I found that FLX flies were becoming larger even though they were collected as virgins, which I assumed would select for a shorter developmental time. I investigated growth rate using the size data from generation 18 and found a significant effect of genotype treatment on growth rate ($P < 2.2e^{-16}$, Figure 2.8). I found that the larger flies also had a shorter development time, which is inconsistent with results from Prasad *et al.* (2000), who showed that selection for faster development time reduced growth rate.

During cultivation I observed that experimental female abdomens appeared to become darker and, testing this, I found a significant effect of genotype on the tergite darkness ($P < 2e^{-16}$), and significant differences between the quadratic fitted curves of the five female genotypes ($P < 2e^{-16}$). Results showed that some genotypes had become darker compared to Cwt (Figure 2.9 and Table 2.1); the FLX homozygote and the two control FM treatments grouped together, while the FLX heterozygote was similar to the Cwt.

I found a significant effect of genotype treatment and zygosity on the locomotory activity of flies ($P = 0.0001$, see Figure 2.10 and Table 2.1). While Cwt males were more active than other males, FLX females were the most active female genotype. Across all genotypes, females were in general more active than males,

although the reverse was true for wild-type genotypes (as previously found; Long and Rice, 2007).

Discussion

I hypothesised that enforcing a female-limited evolution of the chrX would ‘feminise’ the genome, resulting in a more female-like phenotype, such as a larger body and therefore increased fecundity. Although fitness results were not significantly different between the sexes at generation 18, there were indications that carrying one or two copies of the FLX chrX could increase female fitness to the detriment of male fitness as predicted. This led me to hypothesise that a further 20 generations would strengthen this pattern; however, I found that the fitness of FLX females and males became more similar than before. This is in contrast to Prasad *et al.* (2007) and Abbott *et al.* (2010) who measured fitness of the same ML evolution populations 60 generations apart and found the same significant result both times. In both cases, males from the ML experimental population had higher fitness than control males, whereas ML females had lower fitness than controls, as would be expected if fitness were sexually antagonistic. Though the evolution experiments are not completely complementary to each other (I limited selection to the chrX while they limited selection to chromosomes X, 2, and 3), I expected my experiments to show the opposite result to these previous experiments. We used the same base population (LH_M) for the evolution experiments and therefore selected on the same or similar sexually antagonistic variation, although in different sexes.

There are a number of reasons why the predicted effects on female and male fitness were not observed. Sexually antagonistic loci are unlikely to be limited to the chrX, even if it is predicted to be enriched for them (Fry, 2010). Therefore, limiting selection to one sex at all genomic loci simultaneously, as in the ML experiments described above, may provide a more powerful test of the theory. It is also possible that the experimental setup of the female fitness assays was not sufficiently sensitive to detect relatively small differences in fitness between the genotypes. This was the motivation for assaying female fitness at different levels of resource (yeast) availability. In this yeast level experiment, I found that some of the different female genotypes appeared to have significantly different fitness optima at different levels of yeast availability.

Another possibility is that the level of sexually antagonistic variation at the start of the experiment was lower than expected, limiting the possible response to selection. One quantitative genetic measure to assess on-going conflict between the two sexes is the intersexual genetic correlation (r_{mf}). A negative r_{mf} for fitness indicates that intralocus sexual conflict is occurring. r_{mf} has been measured at four different time points during the LH_M population history and while the two earlier time points found a negative r_{mf} [$r_{mf} = -0.30$ (Chippindale et al. 2001) and $r_{mf} = -0.42$ (Innocenti and Morrow, 2010)], the later two did not [$r_{mf} = 0.21$ (Collet et al., 2016), and $r_{mf} = 0.14$ (Pennell, 2016)]. Between the study in 2010 (Innocenti and Morrow, 2010) and the study in 2016 (Pennell, 2016) there was also a decrease in overall levels of sexually antagonistic variation from 62.4 to 48.9%. These results indicate that while intralocus sexual conflict seemed to have happened early in the population history of LH_M, it now appears that the conflict has to have been resolved. The previous ML experiments were started at an earlier time point, and therefore may have had more sexually antagonistic variance available to select on than was available in my experiments, producing fitness results that were clearer than those I found.

A third possibility is that the chrX is already close to the female trait optima, meaning that selection would not have much to act on. This is because the chrX spends two-thirds of its time in females, so sexually antagonistic traits would be more exposed to selection in females (Rice, 1984).

An easily observed sexually dimorphic trait in *D. melanogaster* is body size, with females being larger than males. As, Abbott *et al.* (2010) showed limiting selection to males caused body size to decrease in both sexes, I expected that limiting selection to females only would increase body size in both sexes, which was true at both generations 18 and 72. However, previous studies have shown conflicting results for male body size optimum, with evolution experiments showing that the male optimum for body size is to be small (Pitnick et al., 2001; Prasad et al., 2007; Abbott et al., 2010; Pischedda et al., 2012) and studies on natural populations showing that being large is advantageous (Partridge et al., 1987b, 1987a). It is therefore not clear if the respond in body size to the FLX selection is due to on-going conflict or is a consequence of differences in environments and experimental selection pressures. To ensure repeatability of the measurements, only I did the measuring of the flies and I presumed that any bias would be equalised over the four replicated populations.

Collecting flies as virgins did not (on average) change the development time (225-239h) from the standard development time for flies at 25°C (216-228h, Ashburner et al., 2005), but selected flies increased in body size, caused by faster growth rate for FLX females and males compared to Cwt flies. Growth rate is a sexually dimorphic trait, which I found to responded to FLX selection by driving males further away from their optimum while driving females closer to theirs. This is in agreement with results from Prasad et al. (2007) showing decreased growth rate in ML selected flies, resulting in the opposite pattern: males moved closer to their growth rate optimum, but females moved further from theirs. Together, these results indicate that there is an on-going sexual conflict over growth rate in *D. melanogaster*.

Another visible sexually dimorphic trait is melanisation of the *D. melanogaster* abdomen, which is characterised by black melanised stripes at the posterior of each tergite, with the last two tergites in males being completely melanised. Abdominal pigmentation is a highly plastic trait and is greatly affected by developmental temperature, with lower temperatures generating darker phenotypes (Gibert et al., 2000), so any change in pigmentation should be interpreted with caution. Despite this, studies have found a correlation between mating success and melanisation, with darker females being more sexually receptive (Parkash et al., 2011; Singh, 2015). Thus, increased abdominal melanin pigmentation in the FLX homozygote treatment could be due to a correlated response to selection for sexually receptivity in females. Interestingly, the FLX heterozygote treatment group showed a more similar pattern of melanisation to the Cwt treatment than to the FLX homozygote. As the FLX heterozygote carries one FLX and one Cwt chrX, this pattern could be due to dominance of the Cwt chrX. It is therefore possible that the darker FLX homozygote phenotype is due to a recessive pigmentation allele on the FLX chrX, which changed as a correlated response to FLX selection. A likely candidate could be the X-linked gene, *tan*, which is a gene known to be involved in the *D. melanogaster* cuticle pigmentation pathway and leads to a darker phenotype when expressed at higher levels (Gibert et al., 2016).

Long and Rice (2007) found that adult locomotory activity is the target of intralocus sexual conflict, with males being more active than females. This is consistent with previous results showing that less active females were courted less frequently by males (Tompkins et al. 1982). It follows that females would benefit from reduced locomotory activity when interacting with males, as male courtship is

harmful to females (Nandy et al., 2013). Abbott *et al.* (2011) showed that after ~30 generations of an MLX evolution experiment, the locomotory activity of MLX females had increased to levels comparable to that of males. However, in my experiment, even though FLX males did exhibit decreased locomotory activity compared to Cwt that of FLX females increased. This increase could be attributed to differing experimental setup; Long and Rice (2007) assayed flies in mixed-sex groups whereas I assayed females and males separately, potentially missing male effects on female activity. Also, I used females that were mated before the assay and previous experiments have showed that mated females have increased activity (Isaac et al., 2009). This may be due to the additional need for the females to seek out food or resources for subsequently elevated levels of ovulation (Carvalho et al., 2006). Furthermore, by limiting the amount of available yeast during normal fly culture, female-female resource competition is encouraged, potentially selecting for higher levels of locomotory activity. However, as my results are the only ones reporting this pattern I would be reluctant to draw any conclusions in opposition to previously established results.

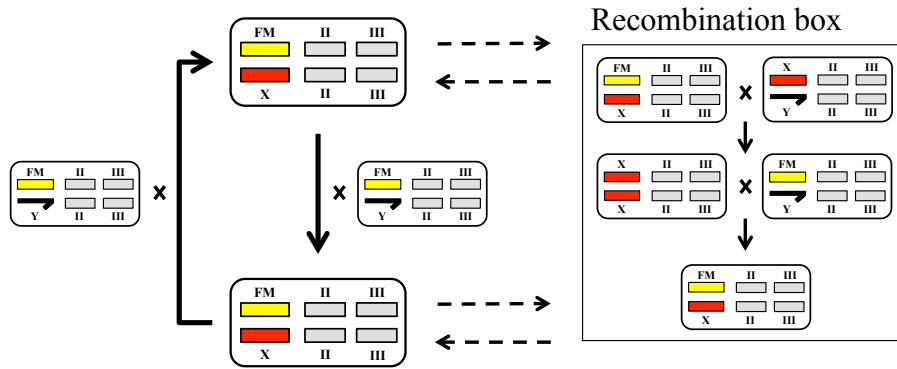
Control FM

To ensure female-limited selection on the chrX I used an FM balancer to prevent recombination with the wild-type (evolving) chrXs. This also allowed me to control how the FLX chrX was inherited from mother to daughter. Because the FM balancer is a modified chrX with one large inversion, the basic assumption is that it functions as a normal chrX, but there might be effects on the flies that carry it, due to the phenotypic markers or other effects of the inversion. The control FM treatment (CFM) was devised to control for the unknown effects of the balancer. The CFM treatment was predicted to respond similarly to the Cwt treatment if the FM balancer functioned like a normal chrX. However, it is clear from the phenotypic assays that this was not the case and that carrying the FM balancer does have an effect on the flies. This is particularly evident from the body size results, where the significant differences in body size between the FLX and CFM treatment groups at generation 18 had disappeared by generation 72. Overall, at the start of the experiment the phenotypic traits expressed by CFM were more similar to Cwt, but at later stages of the experiment CFM is more similar to FLX. This indicates a long-term adaptation to the FM balancer in CFM, which makes it difficult to separate the effect of the FLX

selection from adaptation to the FM balancer in FLX as well. Interestingly, flies perform better after adaptation to the FM balancer. After 40 generations CFM outperformed the other two treatment groups in the fitness assay, and in the yeast stress level assay CFM females had the highest fitness. In conclusion, while it was anticipated that the FM balancer would function as a regular chrX, the inversion and added mutations do seem to negatively affect flies. As the inversion prevents recombination of the FM balancer, this would result in genetic drift and an accumulation of deleterious mutations (Futuyma, 2009). It is possible then that to compensate for the slightly deleterious FM balancer the other chrX and the rest of the genome may adapt to increase overall fitness when in a wild-type state. The CFM chrX passes through males every third generation, removing any harmful male recessive alleles and allowing selection to continue to act on both sexes, as observed in the second fitness assay.

Previous evolution experiments have shown that it is possible to eliminate intralocus sexual conflict by limiting selection to one sex, thereby removing counter selection. These previous studies have been exclusively male-limited, limiting the generality of these tests. Nonetheless, these experiments showed responses that shifted numerous phenotypic traits towards the male optima, causing harm to females that expressed the male-limited genome. To determine if the response to limited selection would be the same in a female-limited experiment as in the male-limited experiments, I performed the first female-limited chrX evolution experiment. I did not find a strong response to selection in most of the phenotypic traits tested, which could be due the chrX already being closer to the female optima. Also, the FM balancer does seem to have a long-term effect on flies, so this experimental setup might not be the best method to estimate a change in sexually antagonistic variation.

Female-limited X-chromosome



Control FM

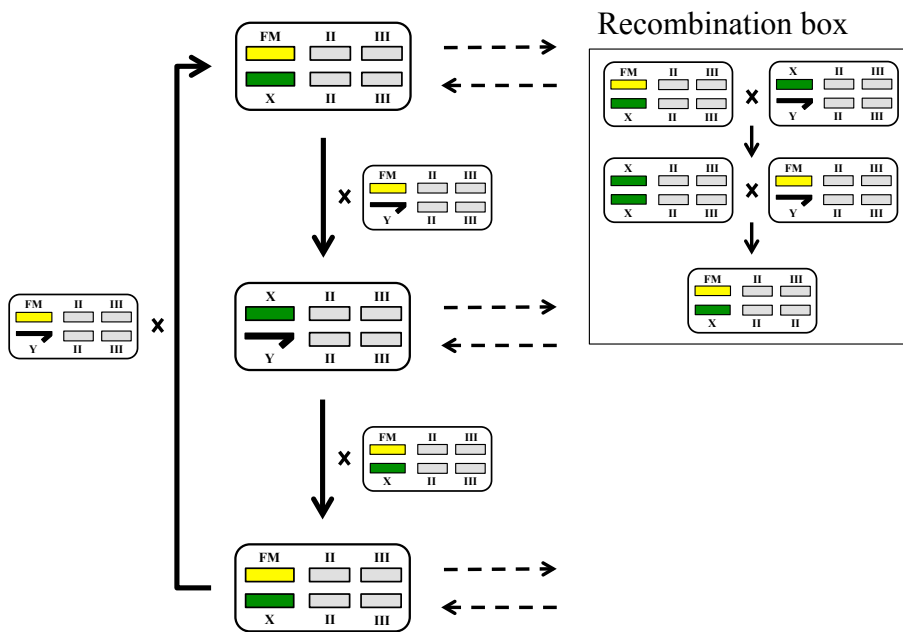


Figure 2.1: Protocol for the Female-limited X-chromosome (FLX) evolution experiment, the FM balancer control, and the recombination box for both. All chromosomes are depicted by different coloured rectangles: light grey (autosome II and III), yellow (FM balancer), red (FLX X-chromosome), and green (CFM X-chromosome). The Y-chromosome is depicted as the black half-arrow. For additional details see *Evolution experiment protocol* in Methods.

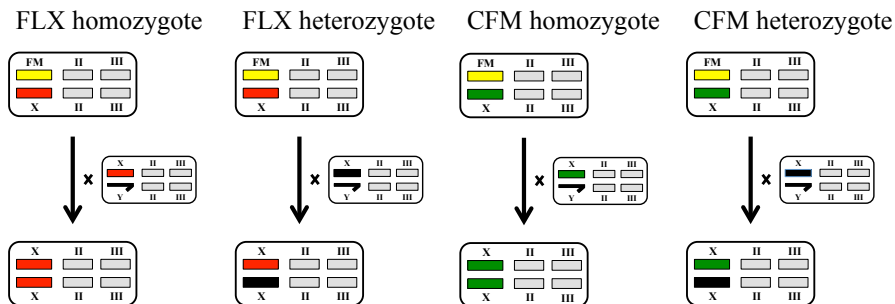


Figure 2.2: The crosses done to get female genotypes without the FM balancer to use in the assay. Genotypes with the same kind of X-chromosome are denoted homozygote (ho), and genotypes with one wild-type X-chromosome are denoted heterozygote (he). All chromosomes are depicted by different coloured rectangles: light grey (autosome II and III), yellow (FM balancer), red (FLX X-chromosome), green (CFM X-chromosome), and black (Cwt X-chromosome). The Y-chromosome is depicted as the black half-arrow.

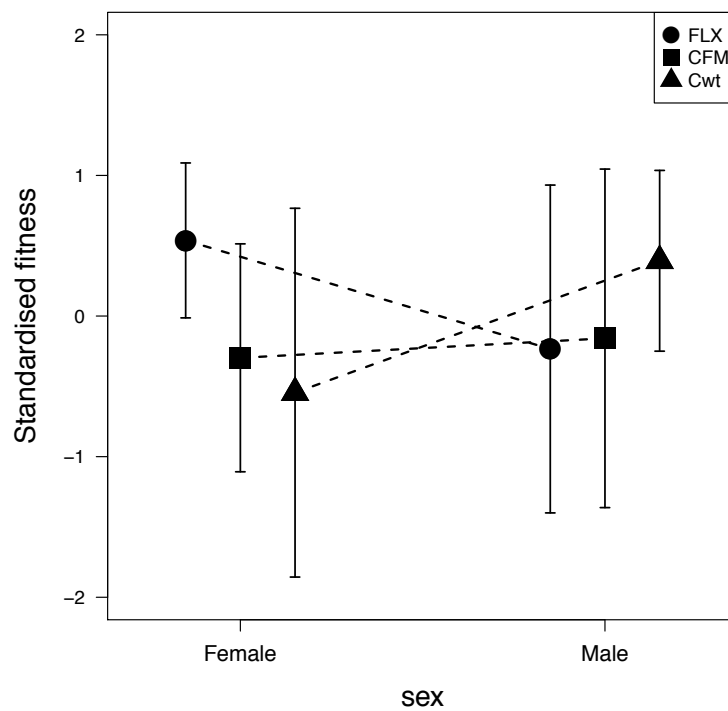


Figure 2.3: Standardised fitness for the three treatment groups at generation 18. A line links the sexes from the same treatment and the error bars are the standard error around the mean. Genotype FLX: circle, CFM: square, and Cwt: triangle.

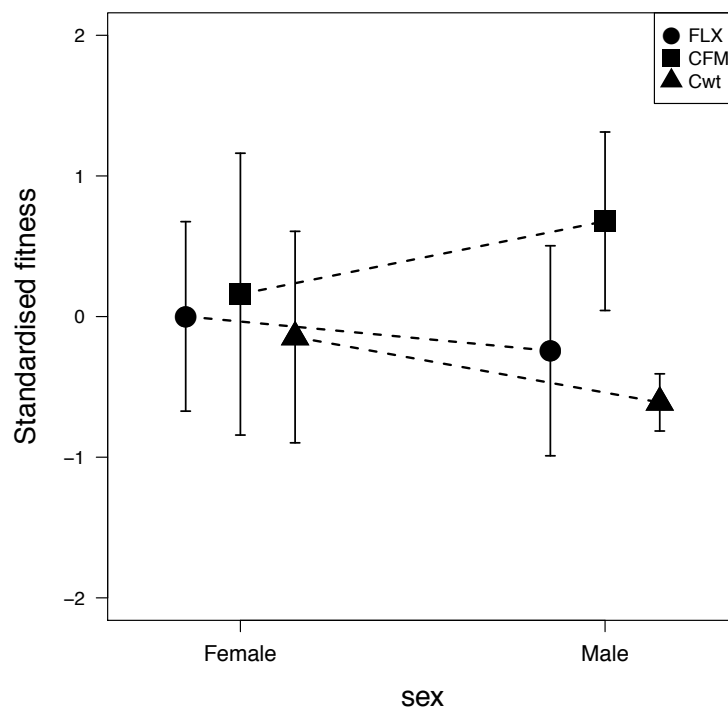


Figure 2.4: Standardised fitness for the three treatment groups at generation 40. A line links the sexes from the same treatment and the error bars are the standard error around the mean. Genotype FLX: circle, CFM: square, and Cwt: triangle.

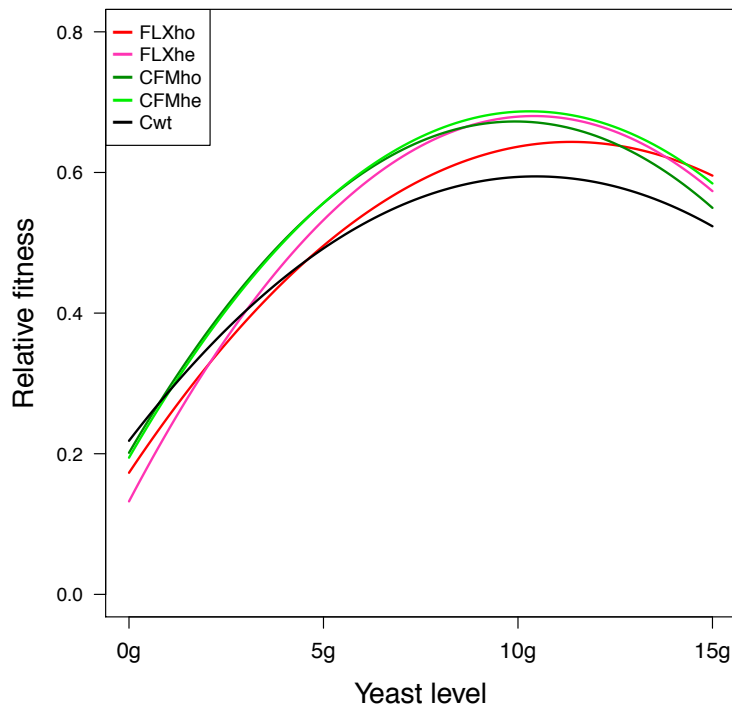


Figure 2.5: Yeast stress assay for the five female genotype treatments. The curves are a fitted quadratic function. Genotype FLX homozygote: red, FLX heterozygote: pink, CFM homozygote: green, CFM heterozygote: light green, and Cwt: black.

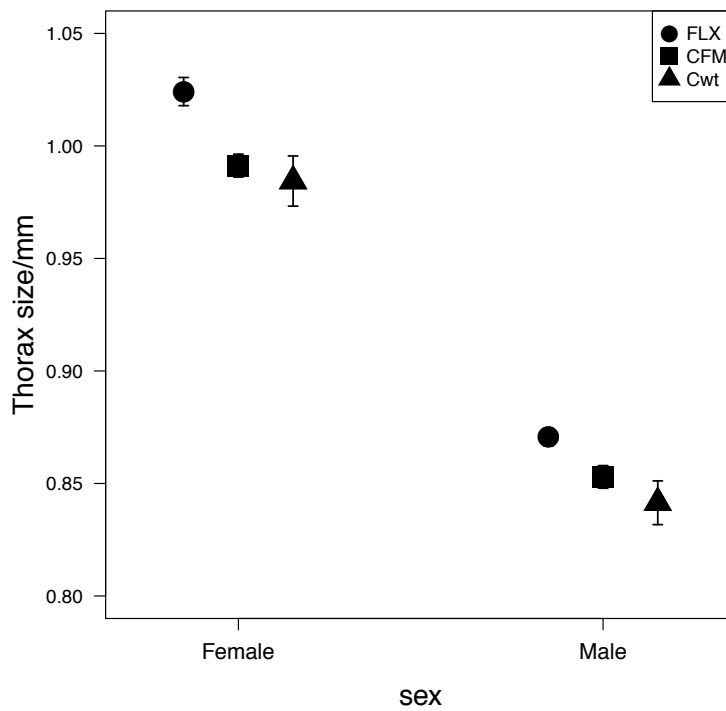


Figure 2.6: Thorax size in mm for the three treatment groups, measured at generation 18. The error bars are the standard error around the mean. Genotype FLX: circle, CFM: square, and Cwt: triangle.

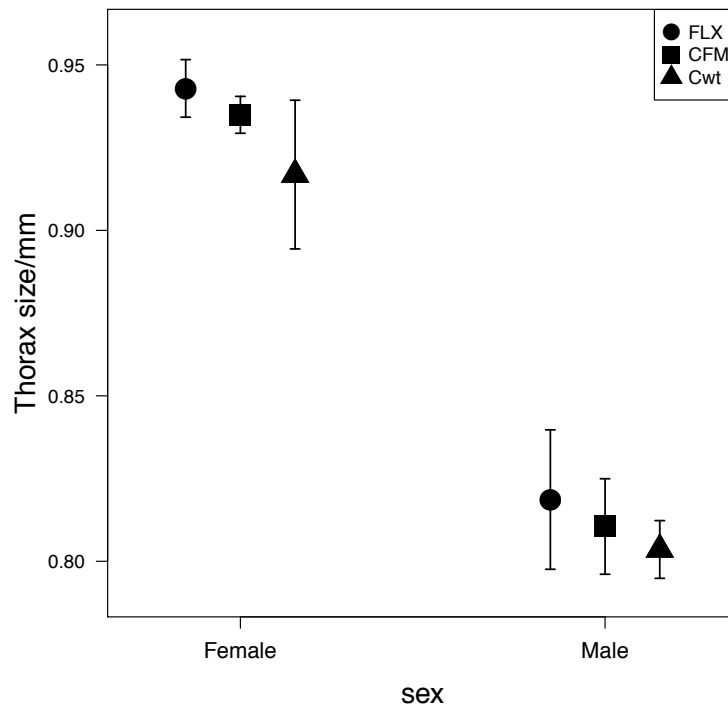


Figure 2.7: Thorax size in mm for the three treatment groups, measured at generation 72. The error bars are the standard error around the mean. Genotype FLX: circle, CFM: square, and Cwt: triangle.

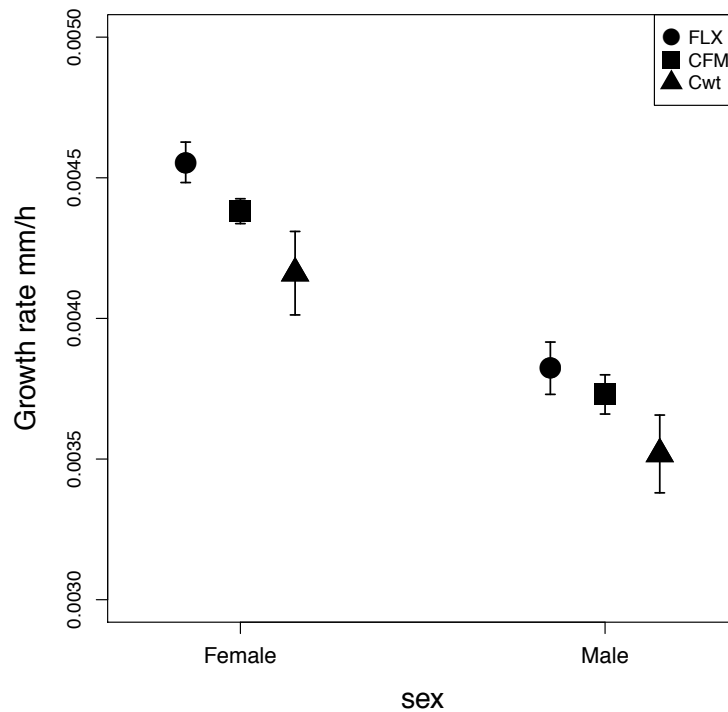


Figure 2.8: Growth rate for the three treatment groups, using size measurements from generation 18. The error bars are the standard error around the mean. Genotype FLX: circle, CFM: square, and Cwt: triangle.

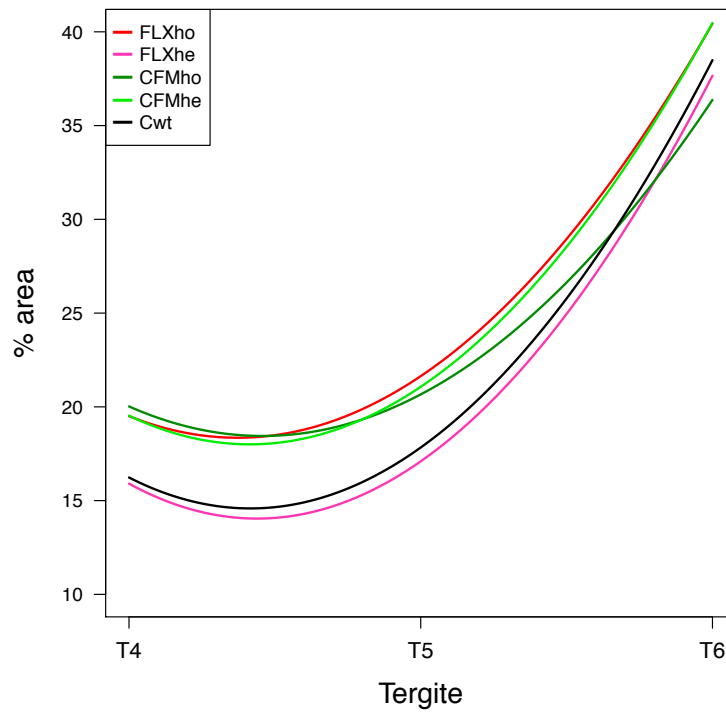


Figure 2.9: The percentage area of dark pigmentation on the last three abdominal tergites for the five female genotype treatments. The curves are a fitted quadratic function. Genotype FLX homozygote: red, FLX heterozygote: pink, CFM homozygote: green, CFM heterozygote: light green, and Cwt: black.

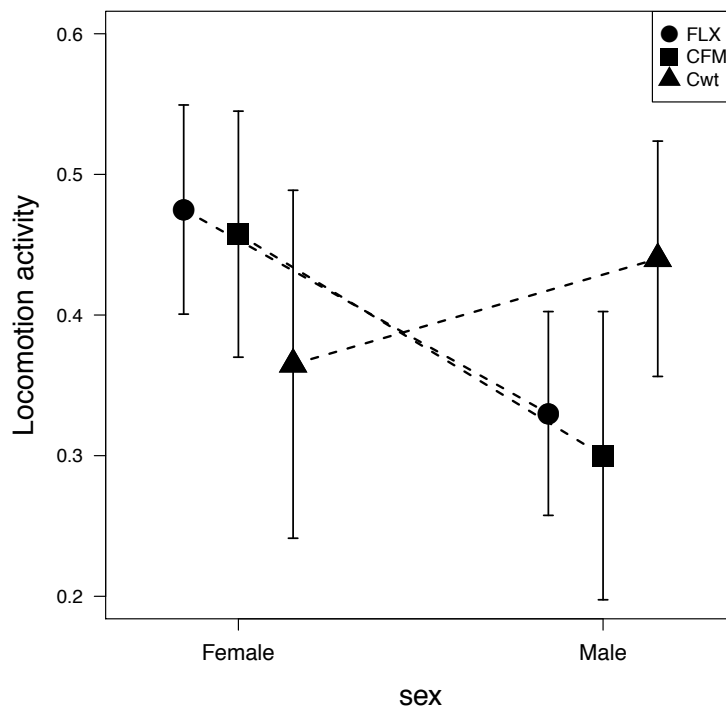


Figure 2.10: Locomotion activity for the three treatment groups. A line links the two sexes from the same treatment and the error bars are the standard error around the mean. Genotype FLX: circle, CFM: square, and Cwt: triangle.

Table 2.1: Summary of results from ANOVA analysis of Linear Mixed-Effects Models.

Source	<i>df</i>	F	χ^2	<i>P</i>
Fitness, generation 18	7		9.00	0.25
Genotype	2	0.87		0.43
Zygosity	2	0.01		0.99
Genotype x Zygosity	3	2.02		0.14
Fitness, generation 40	7		12.42	0.09
Genotype	2	2.92		0.08
Zygosity	2	2.82		0.08
Genotype x Zygosity	3	0.86		0.48
Yeast stress level assay	9		375.75	$< 2.2e^{-16}$
Yeast level	1	512.21		$< 2e^{-16}$
Genotype	4	1.42		0.22
Quadratic term	1	287.38		$< 2e^{-16}$
Yeast level x Genotype	4	2.11		0.08
Thorax size, generation 18	6		149.85	$< 2.2e^{-16}$
Genotype	2	59.10		$2.36e^{-09}$
Zygosity	2	1241.48		$< 2.2e^{-16}$
Genotype x Zygosity	3	2.21		0.12
Thorax size, generation 72	7		102.57	$< 2e^{-16}$
Genotype		4.00		0.03
Zygosity		278.00		$7.77e^{-16}$
Genotype x Zygosity		0.46		0.71
Growth rate	7		91.33	$< 2.2e^{-16}$
Genotype	2	25.37		$2.50e^{-06}$
Zygosity	2	153.41		$2.95e^{-13}$
Genotype x Zygosity	3	0.37		0.77
Abdominal pigmentation	9		180.08	$< 2e^{-16}$
Tergite	1	235.50		$< 2e^{-16}$
Genotype	4	3.91		0.004
Quadratic term	1	302.51		$< 2e^{-16}$
Tergite x Genotype	4	3.30		0.01
Locomotion activity	7		29.70	0.0001
Genotype	2		0.61	0.74
Zygosity	2		11.73	0.003
Genotype x Zygosity	3		16.46	0.0009

Chapter 3: Gene expression of the female-limited X-chromosome in *Drosophila melanogaster*

Abstract

Sexual conflict between males and females occurs when the interests of one sex are in opposition to the other. This can lead to intralocus sexual conflict, where an allele is beneficial to one sex but detrimental to the fitness of the other. To investigate how intralocus sexual conflict affects the transcriptome in both sexes, I carried out an RNA sequencing experiment on a population of *Drosophila melanogaster* that had undergone 50 generations of a female-limited X-chromosome evolution experiment. The data did not indicate a large effect of the evolution experiment on the transcriptome, but this could be because the X-chromosome is already close to the female optimum, meaning that FLX selection would not result in substantial further changes.

Introduction

Sharing a genome between the two sexes can put constraints on each sex due to sexual conflict (Parker, 1979). Sexual conflict can manifest as intralocus sexual conflict (or sexual antagonism) where an allele is beneficial in one sex but harmful in the other (Bonduriansky and Chenoweth, 2009). In particular, it has been theorised (Rice, 1984) and empirically demonstrated that sex chromosomes are enriched for sexually antagonistic genes (Gibson et al., 2002; Innocenti and Morrow, 2010). This is due to the nature of sex chromosomes, where the Y-chromosome (chrY; in male heterogametic species) is only present in males, whereas the X-chromosome (chrX) is present in both sexes. This hemizyosity of the chrX in males allows for an accumulation of recessive male beneficial alleles on the chrX, even if these are detrimental to female fitness. On the other hand, because the chrX spends more time in females, any dominant female beneficial alleles will be selected for, even if harmful to male fitness (Rice, 1984). This makes the chrX a particularly interesting part of the genome for investigating sexually antagonistic variance. Previously, scientists used evolution experiments to investigate the effects of sexually antagonistic variance on Darwinian fitness by limiting selection to either females (Chapter 2) or males (Prasad et al., 2007; Abbott et al., 2010). By limiting selection to

one sex, sexually antagonistic alleles should be free to move towards the optimum for that sex without being held back by selection in the second sex. This is predicted to result in lower fitness in the second sex when it expresses the selected genome, which is what has been shown previously (Prasad et al., 2007; Abbott et al., 2010).

In this experiment, I wanted to investigate how sex-limited selection affected genome-wide patterns of gene expression. For over 50 generations, I conducted a female-limited X-chromosome (FLX) evolution experiment where the chrX was passed from mother to daughter without passing through males, thereby limiting selection on the chrX to females only (Chapter 2). In this chapter, I use RNA-seq (Nagalakshmi et al., 2008) to investigate the transcriptome of the evolved flies and compare it to the wild-type control. I expected that after 50 generation of selection, FLX evolution should be evident as a change in the transcriptome, with females and males expressing the FLX-chrX showing a more ‘feminised’ expression profile.

Methods

Fly stocks

All population treatments are derived from the ancestral wild-type population LH_M, and maintained under standard LH_M culturing protocol at 25°C on a 12-12 light-dark cycle, at 60% relative humidity, and fed on cornmeal-molasses-yeast medium (Rice et al., 2005). On day 12 after oviposition, 16 pairs of adult flies are randomly selected and placed in a food-vial with 6 mg yeast. The pairs are left in the vials for two days to interact, and then transferred to fresh vials without yeast. The females are allowed to oviposit 18 hours, after which all adult flies are discarded. The number of eggs is reduced to 150-200 per vial to maintain constant larval density.

FLX experimental protocol

All treatment groups were kept in four replicated populations, with an adult breeding population of 224 couples (♀16:16♂ per vial) in 14 vials and a minimum of 2,100 offspring in each generation.

Female-limited X-chromosome (FLX) treatment group – To ensure female-limited inheritance of the chrX I used an chrX balancer, FM7a (B^l , sc^8 , v^{Of} , w^a , y^{3ld}). The FM7a balancer (hereafter FM) has all the features of a normal chrX, but cannot recombine. In addition, it also carries special phenotypic markers, which make it easy

to identify and distinguish heterozygotes flies from homozygotes. Heterozygote (FM/X) females were crossed to FM/Y males, so that the chrX would pass from mother to daughter in every generation (Figure 3.1). To control female-limited selection of the chrX, all heterozygote females were collected as virgins.

Control wild type (Cwt) treatment group – As a control for the FLX protocol I kept a wild-type LH_M population under the same experimental conditions, such as virgin collection and a smaller population size.

Control FM (CFM) treatment group – I added an extra control group to assess any unforeseen effects of the FM balancer. CFM was maintained as a wild-type population with the FM balancer present, so the chrX was present in males one-third of the time (Figure 3.1). As with the other groups, all females were collected as virgins.

Recombination box – To ensure that genetic drift and hitchhiking of deleterious mutations did not affect the FLX and CFM chrX I added a recombination box to the experimental setup. At every generation 16 FM/X flies (4%) were removed from the genotype treatments to undergo recombination; 14 FM/X flies (3%) were put back after undergoing recombination (Figure 3.1, Recombination box). Rice (1996) showed that allowing recombination in a small percentage of the experimental population should ameliorate the problems of genetic drift and/or hitchhiking.

RNA extraction

As I only wanted to assay flies without the FM balancer I included two different female genotypes from the FLX and CFM genotype treatment: homozygote (ho), females with two evolving chrXs, and heterozygote (he), females with one evolving and one wild type chrX (Figure 3.2). Thus, I had five female genotypes (FLXho, FLXhe, CFMho, CFMhe, and Cwt) as well as three male genotypes (FLX, CFM, Cwt), giving in total eight different genotypes per replicate population to assay. After 53 generations of experimental evolution, I collected three biological replicate pools of 20 flies per genotype treatment per replicate population, as well as three biological replicate pools of each sex from the original LH_M population; in total 102 samples for RNA extraction. Flies were collected on day 13 after oviposition and left in a vial with food in groups of similar sex and genotype for another 11 days. The flies were quickly anaesthetised using CO₂ and divided into pools of 20 flies and immediately snap-frozen in liquid N₂ before being stored at -80°C. RNA extractions was carried

out using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The samples were sent to AROS Applied Biotechnology (Aarhus, Denmark), where the library preparation (Illumina TruSeq RiboZero) and Illumina HiSeq sequencing were performed according to the Illumina v.4 protocols. For each sample 400ng of total RNA was used, and the samples were run on 9 sequencing lanes with 11 samples in each lane and 1 sequencing lane with 3 samples, which achieved 38-52M reads per sample.

Statistical procedures and bioinformatics

All statistical analyses were conducted in R version 3.2.3 (R Core Team, 2015). Expression data was aligned to the *D. melanogaster* Release 6 reference genome (dos Santos et al., 2015) using the alignment pipeline within the open source software BioConductor (Huber et al., 2015), **QuasR** (Gaidatzis et al., 2015) in R. The data was normalised using the **DESeq** package (Anders and Huber, 2010) from BioConductor and filtered for low variance, resulting in a finale data set of 14439 genes.

To define sex-biased genes, I used the six LH_M samples to estimate differentially expressed genes between male and females. Using the *nbinomTest* in the **DESeq** R package I tested for differential expression between males and females. Genes that were significant for the test were defined as female-biased if they had a negative log2 fold change or male-biased if they had a positive log2 fold change. Genes that were non-significant for the test were defined as unbiased.

I used the generalized linear mixed-effects model for a negative binomial distribution (*glmer.nb*) command from the R package **lme4** (Bates et al., 2015) to analysed the RNAseq data. I used a model including genotype treatment (8 levels: FLXho, FLXhe, FLX male, CFMho, CFMhe, CFM male, Cwt female and male) and zygosity (3 levels: homozygous, heterozygous, or hemizygous for the chrX) as fixed factors and replicate population as a random factor. Significance tests were obtained using the *anova* command for the models that were able to run as genes that had no or very similar expression could not be tested. P-values were adjusted for multiple testing using the *p.adjust* command. Post hoc tests for the model were obtained by the *lsmeans* command from the R package **lsmeans** (Lenth, 2016). To estimate the distribution of genes on the chrX and the number of sex-biased and sexually antagonistic genes I used the *chisq.test* command. To calculate log fold2 change I used *exactTest* from the R package **edgeR** (Robinson et al., 2010), and from the same

package I use *decideTestsDGE* to calculate significantly different expressed genes at $P < 0.05$ adjusting for multiple testing. From the R package **MASS** (Venables and Ripley, 2002), I used *lda* to train a model to recognise sex based on the LH_M samples and used this model to predict the sex of the experimental samples. I used *TukeyHSD* to do a post hoc test of the female/male score within each sex. The Gene Ontology (GO) terms were obtained using the R packages **GenomicFeatures** (Lawrence et al., 2013), **biomaRt** (Durinck et al., 2005), **goseq** (Young et al., 2010), **GO.db** (Carlson, 2016), and **geneLenDataBase** (Young, 2016) from BioConductor.

Results

Out of 14439 genes 13083 could be tested in the model and I found a significant interaction between genotype and zygoty for 212 genes (1.6%). When I looked at the expression of these 212 significant genes in the eight different genotype treatments, there was no clear clustering of replicated populations or genotype treatment within the different genotypes (Figure 3.3).

To investigate if the transcriptome had become more ‘feminised’ I wanted to test if there were an overrepresentation of female-biased genes among the 212 significant genes. However, only 43 genes were classified as female-biased, which was significantly less than in the full set of 13083 genes ($\chi^2 = 10.5$, $df = 1$, $P = 0.001$). Instead 149 genes were classified as male-biased, which was significantly more than in the full gene set ($\chi^2 = 49.9$, $df = 1$, $P = 1.59e^{-13}$). The 14 genes classified as unbiased were significant less than in the full gene set ($\chi^2 = 22.3$, $df = 1$, $P = 2.39e^{-06}$; Figure 3.4).

As the selection pressure was limited to the chrX, I tested if there were an overrepresentation of 212 significant genes located on the chrX. But, I did not find an overrepresentation of the significant genes to be located on the X-chromosome ($\chi^2 = 0.7$, $df = 1$, $P = 0.4$). When I examined the log2 fold change of the female- and male-bias genes on the chrX (Figure 3.5.A) and autosome (Figure 3.5.B) there were not a clear pattern of up- or down-regulation of these genes compared to the Cwt treatment group.

I used the samples of the original LH_M population to train a model to recognise and separate the two sexes, and used this model to determine the sex of the genotype treatments. This gave me a score for how female the different genotype

treatments were. Doing a post hoc test I found no significant difference between the FLXho/he and Cwt females (TurkeyHSD: $P_{FLXho} = 0.81$ and $P_{FLXhe} = 0.59$) or CFMho/he and Cwt females (TurkeyHSD: $P_{CFMho} = 0.11$ and $P_{CFMhe} = 1$, see Figure 3.6.A). I did find a significant difference between FLX/CFM and Cwt males (TurkeyHSD: $P_{FLX} = 0.02$ and $P_{CFM} = 0.0003$, see Figure 3.6.B).

There were 28 post hoc tests between the eight different genotype treatments and the three different levels of zygosity, however not all of these were relevant. Instead, I looked at the post hoc test within each sex, for females (Figure 3.7) and for males (Figure 3.8) for the full set of 13083 genes. To estimate which genes had change during the FLX evolution experiment, I did a GO term analysis of the 698 genes that were significantly different between the FLXho/he and the Cwt/CFMho/CFMhe female genotype treatments and found 26 significant GO terms (Table 3.1). For the 183 genes significant in the post hoc tests for the FLX and the CFM/Cwt male genotype treatments I found seven significant GO terms (**Table 3.2**).

Innocenti and Morrow (2010) previously identified a list of sexually antagonistic genes in the LH_M population. Out of the 212 significant genes I found that six genes matched their list (Table 3.3). This was not significantly more or less than would be expected ($\chi^2 = 2.8$, $df = 1$, $P = 0.09$).

Discussion

After 50 generations of female-limited X-chromosome evolution, I wanted to investigate how patterns in female and male gene expression had changed during the evolution experiment. I used RNAseq to estimate the quantity of RNA in eight different female and male genotype treatments, and was thereby able to compare each sex individually and together. I expected that the FLX chrX would be enriched for female-biased expression, and that FLX males would also be more ‘feminised’ in their expression profiles. However, the results were more complicated to interpret, as they were for the fitness assays (Chapter 2). I found that 212 genes were significant for the interaction between genotype treatment and zygosity (homo-, hetero-, or hemizygous for the chrX). I expected that genotype treatments from the different replica populations would group together as they had experienced the same kind of selection pressure, but I did not find this to be true. It was not possible to determine a pattern of expression for the 212 significant genes as neither genotype treatment, type

of zygosity, nor replica populations grouped together. Instead, as there was no pattern in grouping, these results indicate that, at least, for the 212 genes there was no clear distinction in expression data between the different genotype treatments. Due to the FLX evolution experiment I assumed that genes, which were located on the chrX and beneficial to females, would show a stronger response to the selection, but this was not consistent with the results. Instead, there were significantly fewer female-bias genes among the 212 significant genes than in the full gene dataset, but significantly more male-biased genes. Also, there was no overrepresentation of chrX located genes among the 212 significant genes. As the expression profiles of 212 genes did not group together as expected this could also explain why the results did not match the expectations.

When I looked at sex biased genes from the full data set (13083), which showed significant differences in expression between the Cwt treatment groups and the FLX/CFM treatment groups, I did not see a clear pattern of up- or down-regulation on the chrX or the autosome. But there seemed to be more male-biased genes on the autosomes that had changed expression during the evolution experiment.

Next, I looked at all the genes, 14439, to see if there was an overall ‘feminising’ of the transcriptome in the experimental treatment groups. For the males, both FLX and CFM males were significantly more ‘female’ than Cwt, but for the females there was no change from Cwt, which could indicate that the chrX was already ‘feminised’. As in Chapter 2, the CFM genotype treatments were more like the FLX treatments than the Cwt, indicating again an effect of the FM balancer. To avoid the effects of the FM balancer, I looked at genes that were significant for the interaction between the FLX and the CFM/Cwt genotype treatments. Thus, choosing genes that only had changed under the FLX evolution experiment. For the FLX females, I found 26 significant GO terms, and interestingly water homeostasis was one of the significant terms. Rajpurohit *et al.* (2008) found a possible correlation between body melanisation and water balance, with darker flies showing a higher resistance to desiccation. In Chapter 2, I found that most of the female genotypes (FLXhe, CFMho, and CFMhe) had become darker during the evolution experiment, which could explain why water homeostasis was one of the significant terms. For the FLX males, I found seven significant GO terms.

An important aspect to consider about this RNAseq experiment is the use of whole flies for the RNA extraction, as the method can make it difficult to interpret the

data (Johnson et al., 2013). As, I used whole flies it was not possible to elucidate if any patterns I found were driven by the sex-limited tissue (testes, accessory glands, ovaries and spermathecae). Also, there are many different structures within the whole body, so any specific expression of genes within one structure could be lost when all organs and structures are summed up over the whole body. As I expected the FLX evolution experiment to affect the whole fly, I decided to use the whole body for the RNAseq experiment, though this could mean I might lose specific information about change in gene expression within different structures.

Overall, this experiment did not reveal differences in the transcriptome between the different genotype treatments. This is consistent with previous results that the FLX evolution experiment did not have a large effect on phenotypic traits (Chapter 2). This could be due to selection on the sexually antagonistic variance on the chrX was not strong enough to effect a large change. As there is no pattern to the clustering of the female genotype treatments this could be evidence that the chrX is already close to the female optimum. Thus, the 50 generations of the FLX evolution experiment might not change this much, and the lack of transcriptomic change could be because the chrX is already ‘feminised’.

Female-limited X-chromosome

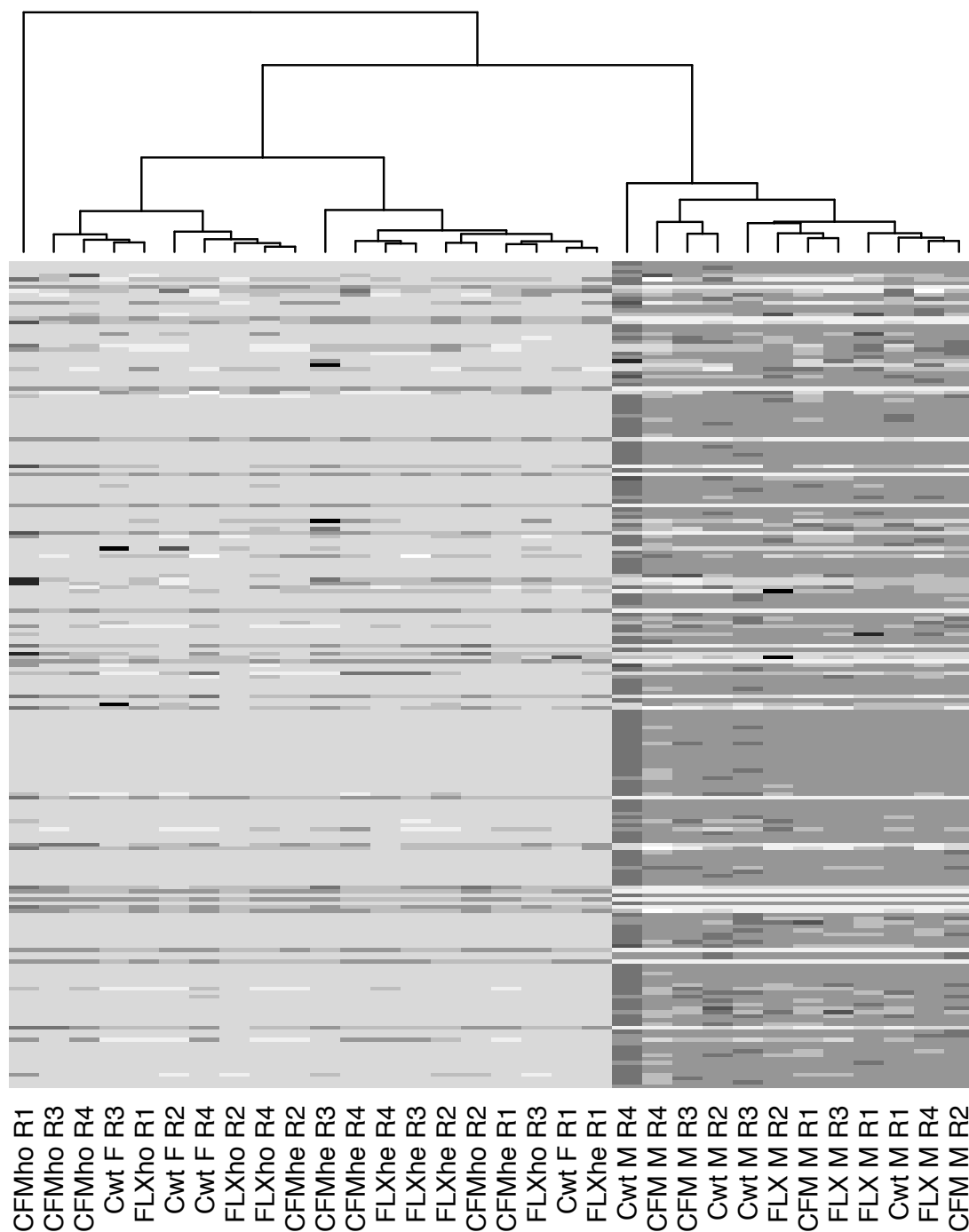


Figure 3.3: Heat map of the expression of the 212 genes significant for the interaction between genotype treatment and zygosity. The colour scale goes from genes with a low expression levels (light grey) to genes with high expression levels (black). M: male, F: female, R: replication population, for annotation see **Figure 3.2**.

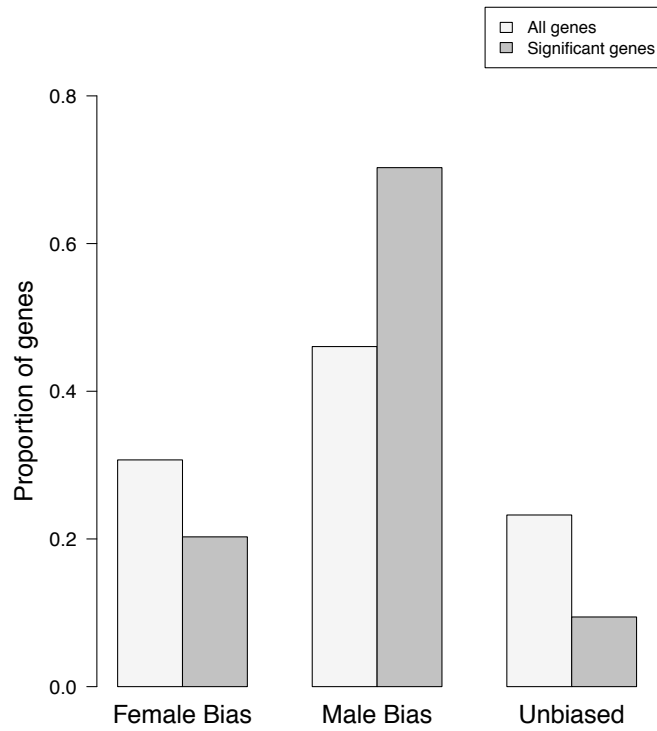


Figure 3.4: The proportion of sex biased genes for either all genes in the full model (light grey) or only for the 212 genes significant for the interaction between sex and genotype treatment (dark grey).

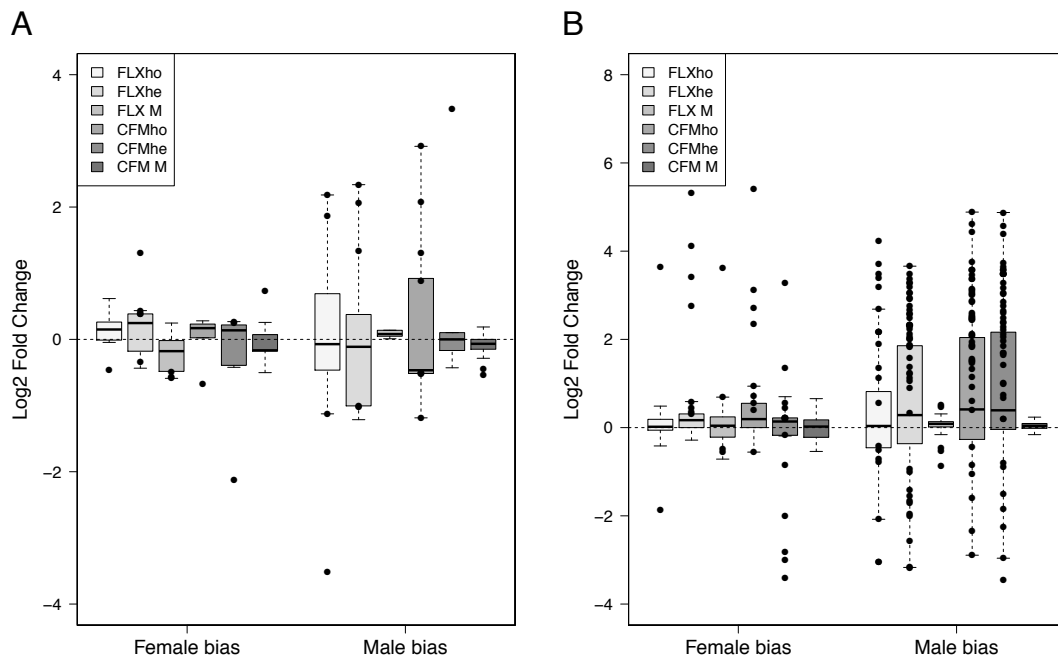


Figure 3.5: Log2 fold change from Cwt for all genotype treatments. A) Genes located on the X-chromosome. B) Genes located on the autosomes. The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles, excluding outliers that exceeded the whiskers. The points are significant differentially expressed genes at FDR < 0.05. For annotation see **Figure 3.2** and M: male.

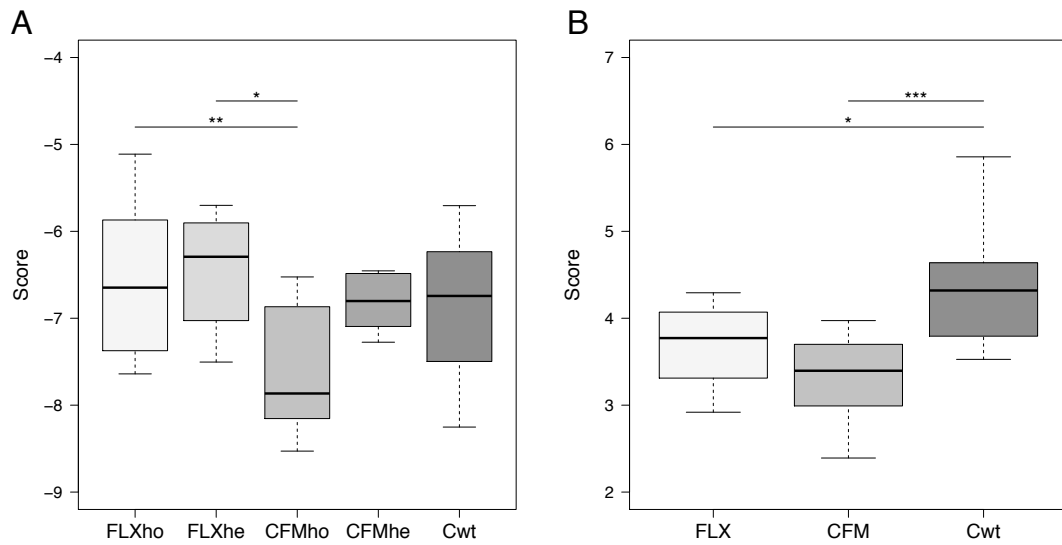


Figure 3.6: Linear Discriminant Analysis for separation of the two sexes, with females being the lower score. A) The five female genotype treatment. B) The three male genotype treatments. The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.05$ ‘*’, $P < 0.01$ ‘**’, $P < 0.001$ ‘***’). For annotation see **Figure 3.2**.

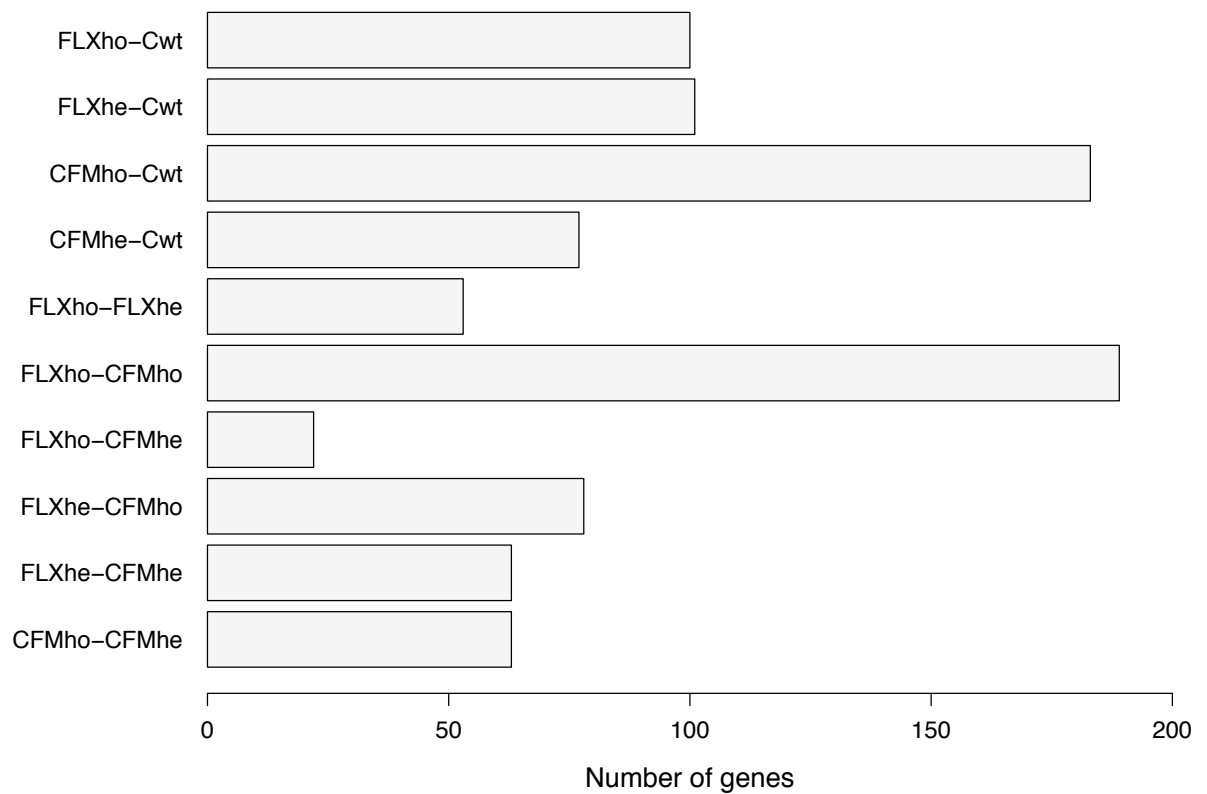


Figure 3.7: Diagram of the genes significant for the post hoc tests between the five different female genotype treatments. For annotation see **Figure 3.2**.

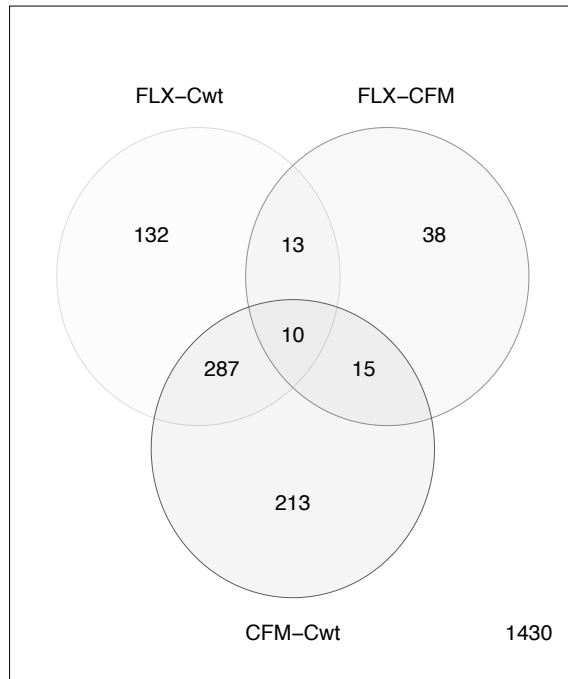


Figure 3.8: Venn diagram of the genes significant for the post hoc tests between the three male genotype treatments.

Table 3.1: GO term analysis of genes significant for the post hoc tests between FLXho/he females and Cwt/CFMho/he female treatments. Only biological processes are listed.

Category	Term	P-value
GO:0006665	Sphingolipid metabolic process	0.0006
GO:0030104	Water homeostasis	0.001
GO:0009617	Response to bacterium	0.002
GO:2000427	Positive regulation of apoptotic cell clearance	0.002
GO:0061357	Positive regulation of Wnt protein secretion	0.002
GO:0006002	Fructose 6-phosphate metabolic process	0.003
GO:0001555	Oocyte growth	0.004
GO:0045116	Protein neddylation	0.004
GO:0006457	Protein folding	0.004
GO:0010972	Negative regulation of G2/M transition of mitotic cell cycle	0.005
GO:0005975	Carbohydrate metabolic process	0.006
GO:0035310	Notum cell fate specification	0.007
GO:0009253	Peptidoglycan catabolic process	0.007
GO:0006289	Nucleotide-excision repair	0.009

Table 3.2: GO term analysis of genes significant for the post hoc tests between FLX males and Cwt/CFM male treatments. Only biological processes are listed.

Category	Term	P-value
GO:0015679	Plasma membrane copper ion transport	0.005
GO:0000462	Maturation of SSU-rRNA from tricistronic rRNA transcript	0.006
GO:0007586	Digestion	0.007
GO:0035235	Ionotropic glutamate receptor signalling pathway	0.008
GO:0007390	Germ-band shortening	0.008

Table 3.3: List of significant genes in the model that were classified as sexually antagonistic.

Gene ID	Official Symbol	Chromosome location
35859	PGRP-SC1a	2R
37213	CG18607	2R
37676	CG9899	2R
39620	Brd	3L
41964	CG8927	3R
42781	CG16723	3R
50458	CG4440	2L
246603	CG30416	2R
2768728	Or46a	2R
5740847	CG17636	X

Chapter 4: Sexually antagonistic coevolution between the sex chromosomes of *Drosophila melanogaster*

Abstract

Antagonistic interactions between the sexes have been proposed to be important drivers of evolutionary divergence. This form of interlocus sexual conflict is modelled as a conflict between alleles at two interacting loci, whose identity remain largely unknown. Here I suggest that if these two loci are located on the sex chromosomes this can lead to a perpetual cycle of antagonistic coevolution between the sex chromosomes and the two sexes. To test this hypothesis I experimentally interrupted the cycle of coevolution by exchanging the sex chromosomes between five wild-type populations of *Drosophila melanogaster* and measured the consequences of these chromosomal replacements for a range of phenotypic and life history traits. I found that disrupting the cycle of coevolution increased male fitness significantly for 10 out of 20 novel genotypes compared to wild-type populations. This could indicate that coevolution happens between the sex chromosomes, and may point to a way genetic divergence could occur between populations and how this could contribute to the evolution of reproductive isolation.

Introduction

The Y-chromosome (chrY) is a unique chromosome in many ways – it is highly heterochromatic, has low gene content and exhibits male-limited transmission. Even though the latest genome assembly of *D. melanogaster* saw a 10-fold increase in assembly size of the chrY, to date only ~10% of the chromosome that has been successfully sequenced (Hoskins et al., 2015). Nonetheless, it has still been possible to estimate the number of genes on the chrY to be around 16 protein-coding genes (Carvalho et al., 2000, 2001; Hoskins et al., 2015; Vibrationovski et al., 2008). As one might expect, most of these genes have male-related functions, and even though the *Drosophila* chrY does not have a sex determining function, it is still essential for male fertility (Carvalho et al., 2001).

In recent years, multiple studies have shown an effect of the chrY on a broader range of phenotypic and life-history traits, such as male fitness (Chippindale

and Rice, 2001), geotaxis (Stoltenberg and Hirsch, 1997), male courtship song (Huttunen and Aspi, 2003), thermal adaptation (Rohmer et al., 2004), suppression of the *sex-ratio* gene on chromosome X (chrX) (Branco et al., 2013; Montchamp-Moreau et al., 2001) and male lifespan (Griffin et al., 2015). These results indicate that variation across different chrYs can have far-reaching consequences for diverse complex and life history traits. However, this is apparently not due to genetic variation, since Zurovcova and Eanes (1999) did not observe any polymorphisms in a protein-coding gene between 11 different *D. melanogaster* chrY. Instead, recent data indicates that this between-chrY variance could be due to epigenetic effects of the chrY (Lemos et al., 2010). Lemos *et al.* (2008) showed that the chrY affected the expression of many non Y-linked genes, and a majority of these genes had a male-biased expression. Since then, Y-linked regulatory variation (YRV) has been shown to control the expression of immunity- and mitochondria-related genes (Lemos et al., 2010) as well as testes-specific genes (Sackton et al., 2011), and silencing of X-linked rDNA in males (Zhou et al., 2012). Furthermore, the genomic background is also apparently important for the epigenetic function of chrY (Jiang et al., 2010). Together, these results indicate that the epigenetic interaction between the chrY and the rest of the genome is important for genome-wide patterns of gene expression, especially genes important for male fitness.

Collectively, these results contradict the standard view that the chrY is a degraded chromosome with no genomic agency. Instead they illustrate how the chrY can interact with the rest of the genome and thereby regulate variation in male fitness. These interactions could be through changing the expression of sexually antagonistic genes - genes where one allele is beneficial when expressed in males, but harmful when expressed female fitness (Bonduriansky and Chenoweth, 2009). However, changing the expression of sexually antagonistic genes could start a cycle of antagonistic coevolution between the sexes, which would follow a similar pattern to the antagonistic coevolution between species (Rice and Holland, 1997). First, any change to the chrY that would increase male-beneficial sexually antagonistic expression should also increase male fitness. This would result in increased female gender-load - the difference between the measured and the optimum trait value for a sex (Rice and Chippindale, 2002). Increase in female gender-load, and the resulting decreased female fitness, would lead to novel selection pressures at other genetic loci and the evolution of counter-adaptions expressed by females to increase their fitness

again. Even though the genome is shared between the two sexes, about 50% of genes in *D. melanogaster* are sex-biased in expression (Jin et al., 2001), and there is an underrepresentation of male-biased (Parisi et al., 2003) but an overrepresentation of female-biased expression on the chrX (Ranz et al., 2003). This ‘feminisation’ of chrX gene expression is consistent with chrX spending two-thirds of the time in females, so selection pressure on the chrX would be greater in favour of females than males. Accordingly, it is likely that decreased female fitness would be counter-acted by female-limited functions on the chrX or epistatic effects of the chrX on the autosomes. This would lead to increased female fitness and the benefit gained by the novel adaption on the chrY would be neutralised. A new adaption would have to occur to increase male fitness again, which would start a new cycle of antagonistic coevolution of the sex chromosomes (Rice and Holland, 1997; Rice and Chippindale, 2002). Thus, over evolutionary time-scales, it would be expected for coadaptation to occur between the sex chromosomes.

To empirically test if the sex chromosomes coevolve in *D. melanogaster*, I designed a round-robin crossing scheme where the coevolution cycle between the sex chromosomes would be disrupted by exchanging sex chromosomes between five wild-type populations. It is expected that sex chromosome coevolution would follow different trajectories in different populations due to random mutations and the interaction between the environment and sexual conflict (Arbuthnott et al., 2014). So by choosing wild-type populations from all over the world with different life histories and adaptations to different climatic regimes, I would expect the sex chromosomes to have followed different paths of coevolution. To ensure I captured as much variation as possible, I selected the five wild-type populations from large, outbred laboratory fly stocks, which should ensure low levels of inbreeding. The round-robin crossing scheme was designed so that one cross between two populations created two novel genotypes where the chrYs from each population were put into the genetic background of the other population (hereafter: novel Y), and a further two novel genotypes where the chrXs were swapped (hereafter: novel X), thus generating all combinations of the novel sex chromosome pairs and autosomal backgrounds. To confirm that any effect I found was due to changing a sex chromosome, I also created novel XY genotypes where both of the sex chromosomes from the same population were placed in alongside with the autosomes from another population. So, if the sex chromosomes coevolve via a process by which variation on the chrY increases male

fitness, and variation on the chrX responds by ameliorating these, then removing either of the sex chromosomes from this pair would release the chrY from any counter adaptations on the chrX, and should increase male fitness. I therefore used male reproductive fitness (the number of live offspring sired by a male) as an estimate of overall male fitness, to confirm if the novel genotypes had disrupted the coevolution between the sex chromosomes.

Methods

Fly stocks

I acquired five outbred lab-adapted wild-type populations sampled from four different continents and three different climatic zones: (1) the *Dahomey* (now Benin, Africa) is a tropical population collected in 1970 (Partridge and Farquhar, 1983); (2) the *Innisfail* stock collected in Australia (Oceania, tropical) in 2012 and founded from 50 females (personal communication, Jennifer Shirriffs); (3) the LH_M stock comes from a larger laboratory adapted population collected in central California (North America, Mediterranean) in 1991 and founded from 400 females (Rice et al., 2005); (4) the *Odder* stock collected in Denmark (Europa, temperate) in 2010 and founded from 589 females (Jensen et al., 2014); (5) the *Tasmania* stock collected in Tasmania (Oceania, temperate) in 2012 and founded from 50 females (personal communication, Jennifer Shirriffs).

All wild-type populations were kept on different culture protocols before arriving to our lab, so to eliminate any environmental difference this might cause I maintained all wild-type populations under the standard LH_M culturing protocol. Thus, all assays could be preformed in a uniform environment, thereby reducing any non-genetic effects on the results. The wild-type populations had been in our lab for at least two generations before the cross. Under the LH_M culturing protocol flies are maintained at 25°C on a 12-12 light-dark cycle, at 60% relative humidity, and fed on cornmeal-molasses-yeast medium (Rice et al., 2005). On day 12 after oviposition, 16 pairs of adult flies were randomly selected and placed in a food-vial with 6 mg yeast. Flies were left in the vials for two days, and then flipped into fresh vials without yeast. The females were allowed to lay eggs for 18 hours, after which all adult flies were discarded. The number of eggs was reduced to 150-200 per vial to maintain constant larval density. Each wild-type population was kept at a size of 640 breeding

adults and a minimum of 3000 juveniles (i.e. within 20 vials).

Preparation of the novel X and Y genotypes

The five wild-type populations were crossed in a round robin-scheme where one population was crossed to two other populations (see Figure 4.1). Since not all possible combinations were used, population pairs were chosen on the basis of belonging to different continents and/or different climates. Pairings were performed reciprocally, generating four novel genotypes for each population, giving a total of ten novel X and ten novel Y genotypes (see Figure 4.1). The crosses were synchronised such that male fitness could be assayed simultaneously for all 20 genotypes.

To generate the novel sex chromosome genotypes I used clone-generator (CG) females with a double-X-chromosome (DX), a Y-chromosome, and two translocations of chromosome II and III (C(1)DX, *y, f*; T(2;3) *rdgC st in ri p^P bw^D*). To illustrate the principle of the entire crossing scheme I will employ the use of two hypothetical wild-type populations A and B:

Novel Y - Cross 1: CG females were crossed to A males (Figure 4.2, 1). This cross was performed with 672 breeding adults (♀32:16♂ per vial) in 14 vials and flipped once without reducing egg numbers. **Cross 2:** Heterozygote females (DX-T(2;3)/A) were crossed to wild-type A males a second time to produce the DX-A females required for crosses 3 and 4 in the novel X crossing scheme (see below; Figure 4.2, 2). This cross was performed with 640 breeding adults (♀16:16♂ per vial) in 20 vials and flipped twice without reducing egg numbers. **Cross 3:** Heterozygote females (DX-T(2;3)/A) were crossed to B males (Figure 4.2, 3), with 320 breeding adults (♀16:16♂ per vial) in ten vials and flipped twice without reducing egg numbers. **Cross 4:** B females were crossed to heterozygote males (T(2;3)/B) bearing a chrY from A (Figure 4.2, 4), with 224 breeding adults in seven vials without reducing egg numbers. This creates ‘novel Y’ males, for use in subsequent assays, where Y-chromosomes from A have been placed in a B genetic background (Figure 4.2, 5).

Novel X - Cross I: CG females were crossed to B males (Figure 4.2, I), with 672 breeding adults (♀32:16♂ per vial) in 14 vials and flipped once without reducing egg numbers. **Cross II:** Heterozygote females (DX-T(2;3)/B) were crossed to B males (Figure 4.2, II), with 640 breeding adults (♀16:16♂ per vial) in 20 vials and flipped twice without reducing egg numbers. **Cross III:** DX-A females (from cross 2 in novel

Y) were crossed to heterozygote males (T(2;3)/B) (Figure 4.2, III), with 320 breeding adults ($\text{♀}16:16\text{♂}$ per vial) in ten vials and flipped twice without reducing egg numbers. *Cross IV*: DX-A females (from cross 2 in novel Y) were crossed to heterozygote males (T(2;3)/A) bearing a chrX from B (Figure 4.2, IV). This cross was performed with 224 breeding adults ($\text{♀}16:16\text{♂}$ per vial) in seven vials without reducing egg numbers. This creates ‘novel X’ males, for use in subsequent assays, where X-chromosomes from population B have been placed into a population A genetic background (Figure 4.2, V).

In both crossing schemes it should be noted that since there is no balancer for small dot chromosome IV, I could not control how it was inherited. The dot chromosome only makes up 1% of the total genome and should thus have a limited effect (Adams et al., 2000). In any case, since the dot chromosome is almost non-recombining (Arguello et al., 2010), at the end of the novel Y crosses the probability of the dot chromosome being from the corresponding autosome population is 0.4. For the novel X crosses the probability is 0.5, and any effects of chromosome IV origin would be averaged out across the multiple individuals assayed.

Sex chromosome-autosome interactions

To estimate the effect of changing the autosomal background when both sex chromosomes were transferred together (i.e. estimating the magnitude of interaction effects between the sex chromosomes and autosomes rather than between the X and Y), I performed crosses using the chosen wild-type populations to create novel genotypes where X and Y-chromosomes from one population are placed into a genetic background of the two largest autosomes from a second population. As with the novel X/novel Y experiment, crosses were synchronized so that male fitness could be assayed simultaneously for all genotypes. Again, I illustrate the crossing scheme using two hypothetical populations A and B.

Novel XY - Cross 1: DX-A females were crossed to A males with a chrY from B (Figure 4.5, 1), with 224 breeding adults ($\text{♀}16:16\text{♂}$ per vial) in 7 vials. *Cross 2*: DX-A females with a chrY from B were crossed to A males with a chrX from B (Figure 4.5, 2), with 448 breeding adults ($\text{♀}16:16\text{♂}$ per vial) in 14 vials. This creates males with both sex chromosomes from B but autosomes from A (Figure 4.5, 3). The probability of the dot chromosomes being from the same population background as autosome II and III was in this case 0.7.

Male fitness of the novel XY genotypes were assayed following the same procedure as outlined above. This assay was done in 10 replicates per genotype.

Male fitness

I used male fitness as an approximation for the overall effect of introducing a novel sex chromosome, since males are where both sex chromosomes are present at the same time. Male fitness was measured as the proportion of live offspring sired by males of the target genotype. Five adult target males (day 12 after oviposition) were combined with ten competitor males and 15 virgin females in a yeasted vial. The competitor males and females were from the outbred LH_M population and homozygous for the visible brown eye (*bw*) genetic marker (LH_M-*bw*) that is recessive to the wild-type red eye-colour allele. After two days, 14 females were isolated in single test-tubes with food media for 18 hours to lay eggs. After the females were discarded, the test-tubes were left under standard LH_M conditions for 12 days and the adult offspring from each tube were counted and scored by eye-colour to assign paternity between target and competitor males. The wild-type red-eye colour allele is dominant to the *bw* allele, so all red-eyed offspring can be assigned to the target males. The male reproductive fitness assay for the novel genotypes was carried out in two blocks, with seven replicates in each for all 25 genotypes, providing fitness estimates based on data from 70 individuals per genotype. For the sex chromosome-autosome interactions, the male reproductive fitness assay was carried out in 10 replicates per genotype, providing fitness estimates based on data from 50 individuals per genotype. Relative fitness was calculated by dividing the fitness for each replicate by the maximum fitness across all replicates.

Change in fitness

To estimate the effect of introducing novel sex chromosomes into the wild type populations, the change in relative fitness was calculated as: $\Delta\text{Fitness} = \omega_{\text{novel genotype}} - \omega_{\text{wild type}}$. All novel genotypes with 95% confidence intervals significantly different from zero and their corresponding wild type populations were chosen for further experiments to investigate the effect in greater details. These genotypes are termed the chosen genotypes in the sections below. Furthermore, a subset of these genotypes was also chosen for Chapter 5. The chosen genotypes were kept in two replicated

populations of 160 breeding adults in 10 vials each. The novel X genotype males were crossed to DX females, to ensure the coevolution of the sex chromosomes in the males, as in the novel Y genotypes.

Phenotypic assays

Male thorax size

Body size was estimated using measurements of thorax length. On day 12 after oviposition, 50 males from the chosen genotypes were placed in 95 % ethanol. Air-dried males were measured using a Nikon SMZ800 dissecting microscope at 63x magnification fitted with an eyepiece graticule.

Courtship rate

Five LH_M female virgins were collected on day of eclosion (day 10 after oviposition) and paired with five target males from the chosen genotypes on day 12 after oviposition. After 48 hours, the five pairs were flipped into fresh vials and observed ten times every half hour throughout the day. The number of males that engaged in courtship during each observation was noted and the rate of courtship was determined as the total number of courtship observations over the ten observations. This assay was done in ten replicates per genotype.

Sperm competition

I looked at two different aspects of sperm competition: sperm defence and offence. Sperm defence (P1) is defined as the ability of one male's sperm to resist displacement by sperm from subsequent matings and is determined by the share of paternity achieved by the first male. To assay P1, five LH_M-*bw* virgin females were collected on the day of eclosion (day 10 after oviposition) and crossed to ten males from the chosen genotypes on day 12 after oviposition. The pairs were allowed to interact for one hour, after which the males were discarded and the females remained in the vial for a further 48 hours. Next, ten LH_M-*bw* males were introduced to the females and allowed to interact for 24 hours. The females were then isolated in single test-tubes and allowed to lay eggs for 18 hours. The females were then discarded and the test-tubes were left under standard LH_M conditions for 12 days. The adult offspring of the five females were counted and scored for eye-colour to assess paternity. Sperm offence (P2) is defined as the ability of one male's sperm to displace

sperm from previous matings and is determined by the share of paternity achieved by the last male. To assay P2, five LH_M-bw virgin females were collected on the day of eclosion (day 10 after oviposition) and crossed to ten LH_M-bw males on day 12 after oviposition. The pairs were allowed to interact for one hour, after which the males were discarded and the females remained in the vial for 48 hours. Next, ten males from the chosen genotypes were introduced to the females and allowed to interact for 24 hours. The females were then isolated in single test tubes and allowed to lay eggs for 18 hours. The females were then discarded and the test-tubes were left under standard LH_M conditions for 12 days. The adult offspring of the five females were counted and scored for eye-colour to assess paternity. This assay was done in three blocks with five replicates for both sperm defence and offence per genotype. I created a new set of the chosen genotypes and repeated the P2 assay at an earlier generation than the first P2 assay. It was performed in the same way, but in two blocks with seven replicates in each.

Male effect on female fecundity

Male effect on female fecundity was measured as the number of eggs laid by the female during an 18 hour period, which corresponds to the period normally available to females from the base population (Rice et al., 2005). Five LH_M virgin females were collected on the day of eclosion (day 10 after oviposition) and combined with five males from the chosen genotypes on day 12 after oviposition. After two days the five females were isolated into single test-tubes for 18 hours to lay eggs and were then discarded, while the test-tubes were frozen so the eggs could be counted at a later date. The fecundity for each female was averaged across the number of eggs laid by the five females. This assay was performed in nine replicates per genotype. Relative fecundity was calculated by dividing the fecundity for each replicate by the maximum fecundity across the whole assay.

Male longevity

On day 12 after oviposition, five males from the chosen genotypes were collected in a food-vial. Mortality was scored daily, six days a week until 95% of the flies were dead. The flies were flipped into new food-vials every fourth day, without replacing dead flies, so that density was free to decline over time. The flies were maintained

under standard LH_M conditions until death. This assay was performed in ten replicates per genotype.

Female longevity with male harassment

Five LH_M virgin females were collected on day of eclosion (day 10 after oviposition) and crossed with five target males from the chosen genotypes in a yeasted vial on day 12 after oviposition. Mortality of the females was scored daily, six days a week until 95 % of them were dead. The flies were flipped into new food-vials with yeast every third day. At every flip, new males and LH_M-*bw* females were added to the vials to replace any dead flies and keep the sex ratio equal. The flies were maintained under standard LH_M conditions until death. This assay was performed in ten replicates per genotype.

Offspring egg-to-adult viability assay

Offspring egg-to-adult viability was estimated as the proportion of 100 eggs that developed into live adults within 12 days. 20 pairs of flies from the chosen genotypes were placed in bottles with a cornmeal-molasses-yeast medium plate overnight to oviposit. 100 eggs were counted and transferred to a fresh food-vial, and left under standard LH_M conditions for 12 days. The number of pupae, dead and live flies was counted as well as the sex of the adult flies for the sex ratio assay. This assay was performed in two blocks with five replicates in each block.

Statistical procedures

All the statistical analyses were conducted in R version 3.2.3 (R Core Team, 2015). I used the Generalized Linear Models (*glm*) command with genotype as a fixed factor to test male fitness from the sex chromosome-autosome interactions assay, courtship rate, male effect on female fecundity, and thorax size. Significance tests were obtained using the *anova* command in R. For the male reproductive fitness assay I used the Linear Mixed-Effects Model (*lmer*) command from the R package **lme4** (Bates et al., 2015). Due to collinearity between population and genotype in the male fitness assay, I could not use a single model with both of these factors included. Instead I fitted two separate models with either genotype or population as a fixed factor. For both models was experimental block included as a random factor. I also used *lmer* for the egg-to-adult viability, sex ratio assays, and sperm competition.

Significance tests were also obtained using the *anova* command. The 95% confidence intervals around $\Delta\text{Fitness}$ was calculated by bootstrap, randomly resampling 13 out of 14 data points and recalculating $\Delta\text{Fitness}$ 10,000 times. I also used the *binom.test* command to test the $\Delta\text{Fitness}$ estimates. To calculate male and female longevity I used the **survival** package (Therneau and Lumley, 2016), the *survfit* function was used to create survival curves, and the *survdiff* to test for difference between the survival curves.

Results

I created 10 novel X and 10 novel Y populations to test if the sex chromosomes in *D. melanogaster* coevolve, see Figure 4.1 and Figure 4.2. When modelling male fitness with population as a fixed factor, I found that there was a significant effect on male fitness ($P = 6.452e^{-11}$, see Figure 4.3 and Table 4.1). Post-hoc tests across novel X populations showed that males with a novel X within *Innisfail* or *Odder* backgrounds had significantly higher fitness than their wild-type counterparts (Figure 4.3.A). Males with a novel Y from LH_M or *Innisfail* had significantly higher fitness than the *Innisfail* wild-type (Figure 4.3.B). When modelling male fitness with genotype as a fixed factor there was also a significant effect on male fitness ($P = 0.0002$, Table 4.1). Males with a novel X or Y chromosome had significantly higher fitness than wild-type males (Figure 4.4). To exclude interactions between the novel sex chromosome and the autosomes, I created novel XY genotypes (Figure 4.5). I found that males from the novel XY genotypes did not have significantly different fitness from the wild type genotypes ($P = 0.71$, see Table 4.1 and Figure 4.6). It therefore seems that introducing a new sex chromosome in isolation resulted in the difference in fitness. To investigate this in further detail, I calculated the difference in fitness between the novel genotypes and their wild-type counterparts. I found that four of the novel X populations (I-L_X, I-O_X, O-I_X, and O-D_X) were significantly different from zero, as well as six of the novel Y populations (L-I_Y, I-L_Y, I-O_Y, O-I_Y, O-D_Y, and D-O_Y; see Table 4.2 and Figure 4.7). Interestingly, 16 out of the 20 point estimates were positive, which is significant ($P = 0.01$) using a binomial test. So, with half of the novel genotype having confidence intervals not overlapping 0 and a significant majority being positive, my data shows that, in general, changing a sex chromosome has a significant positive effect on male fitness.

To study the implications of the higher fitness on males and their mates I chose the ten novel genotypes that had had the largest effects and their four corresponding wild type populations for all future assays.

Direct effects

I was interested in examining which phenotypic traits, other than male fitness, could be affected by the introduction of a new sex chromosome and how these could be related to male fitness. I began with male body size, as it has previously been shown that body size is an important factor in male fitness (Pitnick, 1991). I found that there was a significant difference in size between the novel and wild-type genotypes ($P = 3.56e^{-09}$, Table 4.1). In fact, the males from the novel X genotypes were significantly larger than both wild-type males ($P < 1e^{-04}$) and novel Y males ($P < 1e^{-04}$). However, the males from the novel Y genotypes were not significantly different from wild-type males ($P = 0.70$, see Figure 4.8). It is therefore possible that a larger body size for some of the novel genotypes could account for the increase in fitness.

I then examined male lifespan, since chrYs have been shown to affect male lifespan (Griffin et al., 2015). However, I did not find any difference in lifespan between the different treatment groups ($P = 0.96$, see Table 4.1 and Figure 4.9).

Because the novel genotypes had a higher fitness, I wanted to investigate whether this was reflected in behavioural differences such as courtship activity. I found a significant difference in courtship rate between the different genotypes ($P = 0.0001$, Table 4.1). However, wild-type males had significantly higher courtship rates than males from either of the novel sex chromosome genotypes (novel X: $P = 0.0001$, novel Y: $P = 0.03$), which did not differ from one another ($P = 0.08$; see Figure 4.10).

Since the novel genotypes were less active during courtship, but still had higher fitness, the gains in fitness may instead be achieved through advantages in postcopulatory sexual selection, such as sperm competition. In terms of sperm defence (P1), there were no significant difference in the proportion of offspring produced by the target male between the novel and the wild-type genotypes ($P = 0.24$; see Table 4.1 and Figure 4.11). In terms of sperm offence (P2), there were also no significant difference between the novel and the wild-type genotypes ($P = 0.42$; see Table 4.1 and Figure 4.12).

Indirect effects

I also wanted to investigate which effects the novel genotype males had on their partners' phenotypic traits. Seminal fluid proteins are known to induce ovulation in females (Herndon and Wolfner, 1995), but I did not find any significant difference between the male genotypes in inducing egg-laying in females ($P = 0.18$, Table 4.1 and Figure 4.13).

Interacting with males has been shown to negatively affect female lifespan (Fowler and Partridge, 1989). I did not find a significant difference between the three genotype treatments in how long females lived when continuously exposed to males ($P = 0.18$, Table 4.1 and Figure 4.14), but females still had a shorter lifespan than the males.

Because the total number of offspring (male fitness assay) showed a significant difference between genotypes ($P = 0.02$, Table 4.1 and Figure 4.15), I decided to investigate egg-to-adult viability of the offspring. I found that there was a significant overall effect of genotype on offspring viability ($P = 0.004$, Table 4.1 and Figure 4.16). Specifically, novel X males had significantly lower offspring viability than offspring from wild-type ($P = 0.03$) or novel Y males ($P = 0.004$), but offspring viability of novel Y males did not differ significantly from that of wild-type males ($P = 0.78$). It therefore seems that for novel X males, decreased offspring viability is an indirect cost of higher fitness. I also estimated offspring sex ratio, to see if reduced overall survival was due to low survival rates in male offspring who inherited the novel sex chromosomes. However, there was no significant difference in the proportion of male offspring between the genotype treatments ($P = 0.21$, Table 4.1 and Figure 4.17).

Discussion

It has previously been theorised that an intergenomic conflict would occur between different alleles in different individuals (Rice and Holland, 1997; Rice and Chippindale, 2002). I hypothesised that an intergenomic conflict would be greater between sex chromosomes as they are largely gender-limited and would consequently have a higher degree of opposing interests than the autosomes, which are equally shared between the two sexes. If there is strong intergenomic conflict between the sex chromosomes this could lead to antagonistic coevolution of the sex chromosomes. So,

I wanted to empirically test if the sex chromosomes coevolve in *D. melanogaster*. I did this by using an elaborate crossing scheme where one sex chromosome was introduced into a different sex chromosome and autosomal background, so that the only difference from the original wild-type population was one novel sex chromosome.

I expected that disrupting the cycle of antagonistic coevolution between the sex chromosomes by introducing a novel sex chromosome would increase male fitness. If the cycle of antagonistic coevolution starts with an increase in male fitness, this over time would lead to an accumulation of male-benefit functions on the chrY, with the chrX continually compensating for any harmful effects to female fitness. I predicted that introducing a novel sex chromosome (either X or Y) would lead to an increase in male fitness, since this new pair would come from two different evolutionary paths. So, the new chrX would not possess the same counter-adaptations to the chrY as the original chrX, freeing chrY male-beneficial functions and increasing male fitness.

I crossed five geographically distant populations in a round-robin scheme so one wild-type population was reciprocally crossed to two others and measured the reproductive fitness of the males. I found, as predicted, that introducing a novel sex chromosome significantly increased male fitness compared to the corresponding wild-type populations. So, by releasing the chrY from the counter-adaption of the chrX, all the male-beneficial functions of the chrY are free to increase the fitness of males with no attenuation from the chrX.

Benefits

To investigate which male-beneficial functions could contribute to the increased male fitness found in the novel genotypes, I looked at traits that have been shown to be correlated with male fitness, such as size (Partridge et al., 1987; Pitnick, 1991), courtship rate (Partridge et al., 1987a), and sperm competition (Clark et al., 1999). I found that novel X males were significantly larger than the wild-type males, which is consistent with other results showing that larger males have a mating advantage (Partridge and Farquhar, 1983). Since I did not find any difference between novel Y and wild-type genotypes in male size, it seems that size is not influential to male fitness in novel Y males suggesting that different genetic mechanisms may be

responsible for the increased male fitness exhibited by the two novel genotype treatments.

Unlike previous experiments which showed that larger males have a higher courtship rate and remating rate (Partridge et al., 1987a; Pitnick, 1991), I observed that males from both novel genotypes were less active during courtship. This conflicts with the fitness results I found; I would expect that high fitness males would engage and participate in more matings than low fitness males, but Bedhomme *et al.* (2008) also found lower courtship rate in high fitness males. A possible explanation for the lower courtship rate could be that males become more efficient at courtship thereby decreasing courtship duration or perhaps they outperform other males through sperm competition, and sire a larger percentage of a female's offspring. Bangham *et al.* (2002) found that larger males perform better in sperm competition, but I did not find that males from novel X genotype treatment were better at either P1 or P2 compared to wild-type males. Neither were males from novel Y genotype treatment better at P1 or P2 compared to the wild-type treatment. Overall, I was not able to identify which trait increased the fitness of novel genotype males from the assays done here.

Harm

Introducing a novel sex chromosome into a different genetic background was beneficial for male reproductive success, since the male-beneficial functions of the chrY were no longer constrained by their coevolved chrX. If the interactions between the sex chromosomes were antagonistic, I would assume that these male-beneficial functions would be harmful to females, and so, I wanted to investigate which phenotypic traits were harmful to female fitness. For example, male accessory gland secretions have been found to induce and/or increase female egg production (Eberhard, 1996). It is therefore possible that the higher relative fitness of novel genotype males is due to production of larger or more potent accessory gland secretions, inducing high levels of ovulation and thus causing greater harm. However, I found no evidence that females mated to novel genotype males were more fecund. A second possibility is that female lifespan is compromised following mating with novel genotype males. It has previously been shown that mating with males have a negative effect on female lifespan (Friberg and Arnqvist, 2003; Partridge et al., 1986), and even though my results confirm a reduction in female lifespan after mating with males, I did not find any difference between the treatments. Overall, I found that

males with novel genotypes had higher fitness than wild-type males, but these gains were not achieved by being more harmful to their mates.

Although I could not find any evidence of direct costs to females, it is possible that males would trade-off elevated reproductive success over other life-history traits. One such trade-off that has been well studied is between reproduction and longevity (Williams, 1966); however I found no difference between the lifespans of wild-type and novel genotype males. Prowse and Partridge (1997) have shown that virgin males live significantly longer than males who mate throughout life, and so it is possible that my design, where the males were housed alone, was too ineffective to separate any differences between treatments.

Another possibility is that there is a trade-off between number of offspring and offspring quality. Friberg *et al.* (2011) found evidence of sex-linked paternal effect on egg-to-adult survival. Though I found no difference in number of eggs laid, I did find a significant effect of male genotype treatment on total offspring number. This lower number of adult offspring could be due to decreased egg-to-adult viability. In line with this hypothesis, I did find that novel X males sired lower viability offspring; this is consistent with results showing that offspring sired by larger males have a lower survival rate (Friberg and Arnqvist, 2003). Since there are no known sex ratio meiotic drivers in *D. melanogaster* (Presgraves, 2008), and I found no significant difference in sex ratio, decreased offspring survival is unlikely to be explained by meiotic drive.

If sex chromosomes coevolved antagonistically I would assume that a gain in male fitness would come at a cost to female fitness. I did not find a direct cost to female fitness, but I did find that the offspring of novel X males had reduced egg-to-adult viability. I was not able to establish which harmful effects introducing a novel chrY had on female fitness through the assays performed in this experiment. A possible cost to female fitness not investigated in this experiment is the reduction of female willingness to remate after mating with a novel Y male, which could lead to a reduction in life-time reproductive fitness of females.

In conclusion, I wanted to empirically test if the sex chromosomes in *D. melanogaster* coevolve and found that by changing sex chromosomes between different genetic backgrounds and decoupling the interaction between the sex chromosomes, I was able to establish that this interaction was epistatic and independent of any interaction with the autosomes. However, previous studies using population crosses to examine sexual

conflict have shown conflicting results, as the underlying detailed processes can be difficult to infer from these crosses (Chapman et al., 2003; Rowe et al., 2003). Thus, even though 10 out of 20 genotypes show a significant increase in fitness, only two out of the five wild-type populations were contributing to this effect, and as such these results should be interpreted with caution. Another consideration is the interactions between mitochondria and the nuclear genome of the different novel genotypes. Previous studies in *D. melanogaster* have shown that the interaction between foreign mitochondria and the nuclear genome can be devastating for male fitness (Innocenti et al., 2011; Camus et al., 2012, 2015). The mitochondria are present in both males and females, but are generally inherited through females only. Thus, any response to selection on the mitochondria DNA would occur in females, thereby enabling accumulation of male harming mutations in the mitochondria DNA. This hypothesis has been supported by results which showed that interacting with a foreign mitochondria effected male gene expression in the nuclear transcriptome (Innocenti et al., 2011), aging in males (Camus et al., 2012, 2015), male fertility (Camus et al., 2015), and respiratory capacity (Wolff et al., 2016). As both novel sex chromosomes are expressed with foreign mitochondria, it is worth considering that this interaction could have an effect on male fitness. Though, it is not possible to infer from these assays the magnitude of such an interaction compared to the interaction between chrX and Y.

Overall, I confirmed previous results from Chippindale and Rice (2001) that variation on the chrY is a contributor to male fitness. Also, the empirical test of coevolution between the sex chromosomes indicates that such an interaction is a possibility, which could help further our understanding of how genetic and phenotypic divergence arises between populations and how this can contribute to speciation events.

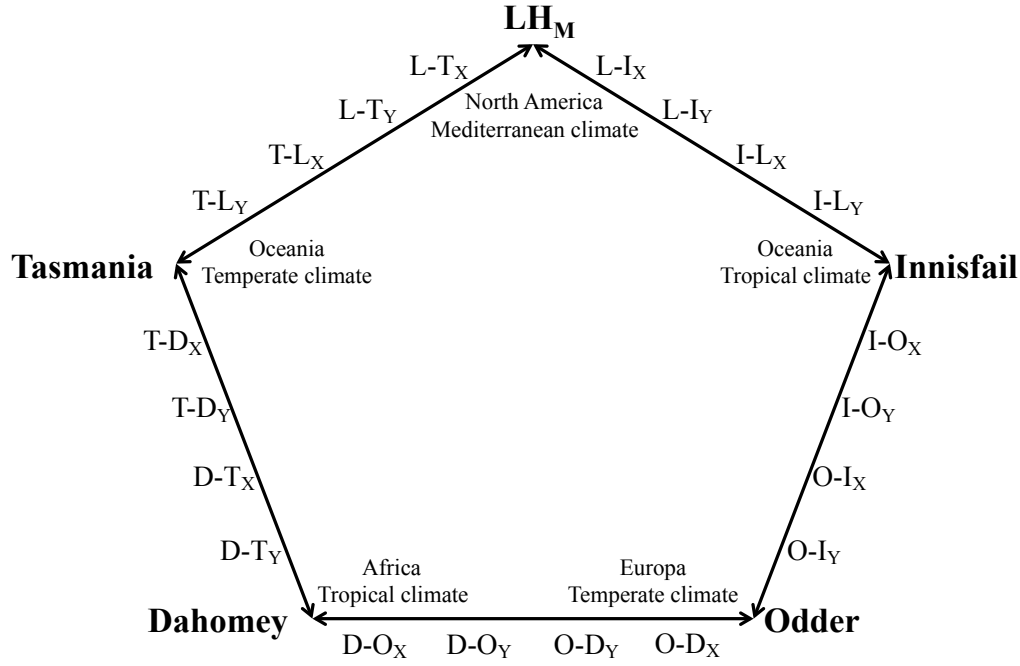


Figure 4.1: Experimental setup. A round-robin design was used where the sex chromosomes from each of the 5 individual wild-type populations were combined with those from two other populations. Each wild-type population was crossed to two populations from different continents and/or climates. Pairings were performed reciprocally, generating four novel genotypes for each population, resulting in a total of ten novel X and ten novel Y genotypes. Annotation for the novel genotypes: First capital letter is the wild-type background with either a novel chromosome X or Y from a different genotype. E.g. $L-I_X$ has a LH_M wild-type background with an X-chromosome from the Innisfail population. D: *Dahomey*, I: *Innisfail*, L: LH_M , O: *Odder*, and T: *Tasmania*.

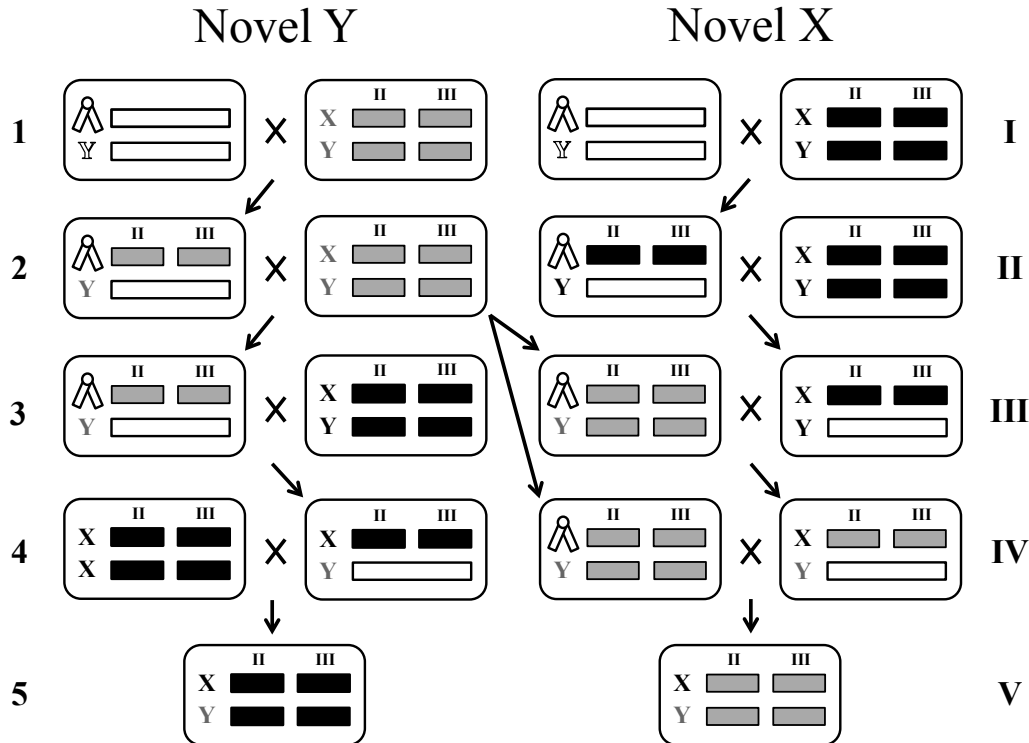


Figure 4.2: Protocol for crosses to create novel genotypes. Sex chromosomes are denoted by letters (X/Y), autosome II and III are depicted as rectangles, and the autosome translocation (2:3) is depicted as the elongated white rectangle. The double-X-chromosome is depicted as ⌘ . Wild-type chromosome is either grey (population A) or black (population B). Only offspring used for the cross in the next generation are shown. For additional details see *Preparation of the novel X and Y genotypes* in Methods.

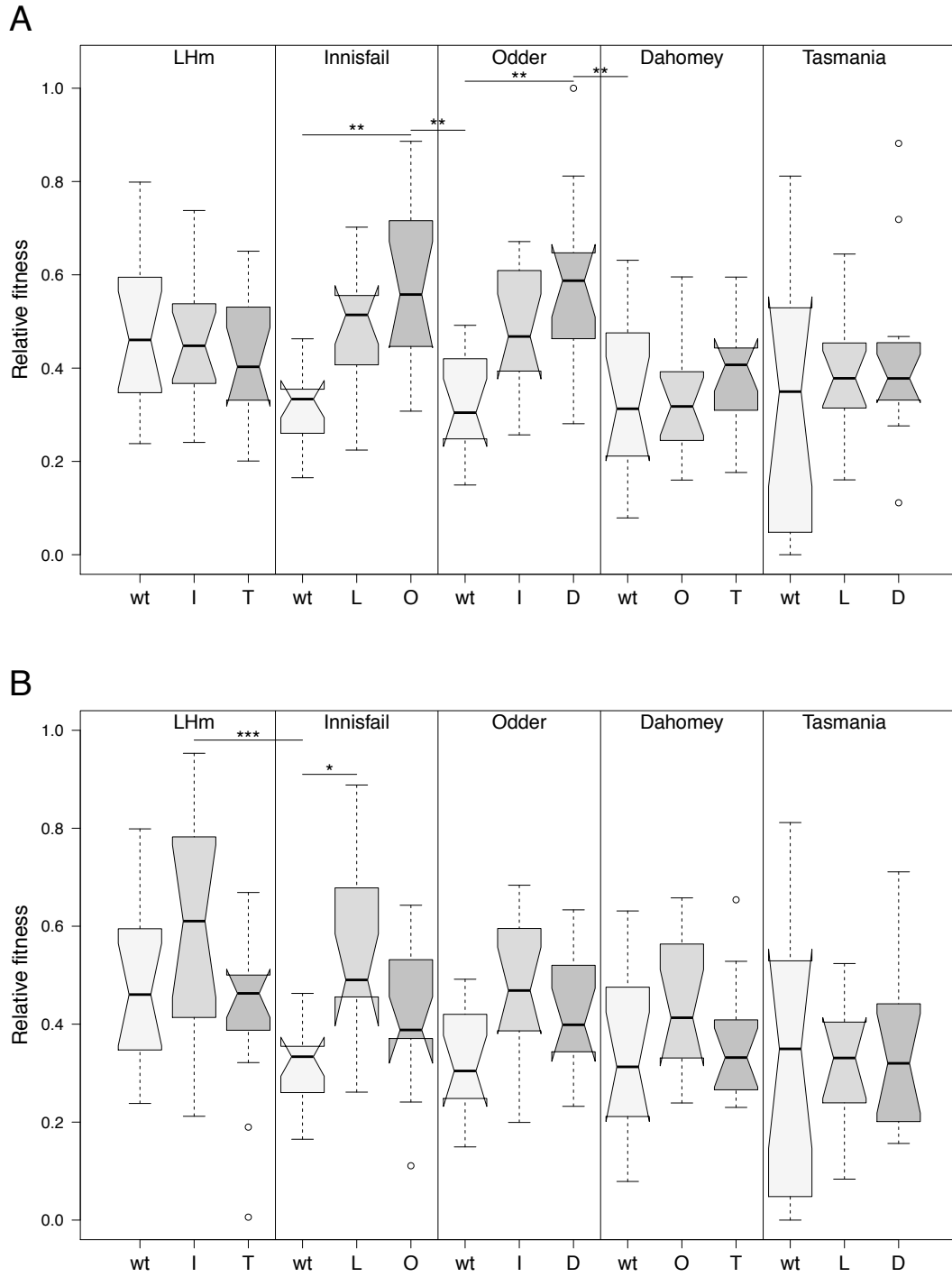


Figure 4.3: Relative male fitness for 20 novel genotypes and five wild-type populations. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. A) Novel X genotypes. Each of the five wild-type genotypes is plotted as reference for the two novel X genotypes in each panel. The capital letter indicates which population the X-chromosome originates from. B) Novel Y genotypes. Each of the five wild-type genotypes is plotted as reference for the two novel Y genotypes in each panel. The capital letter indicates which population the Y-chromosome originates from. D: Dahomey, I: Innisfail, L: LH_M, O: Odder, and T: Tasmania. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.05$ ‘*’, $P < 0.01$ ‘**’, $P < 0.001$ ‘***’).

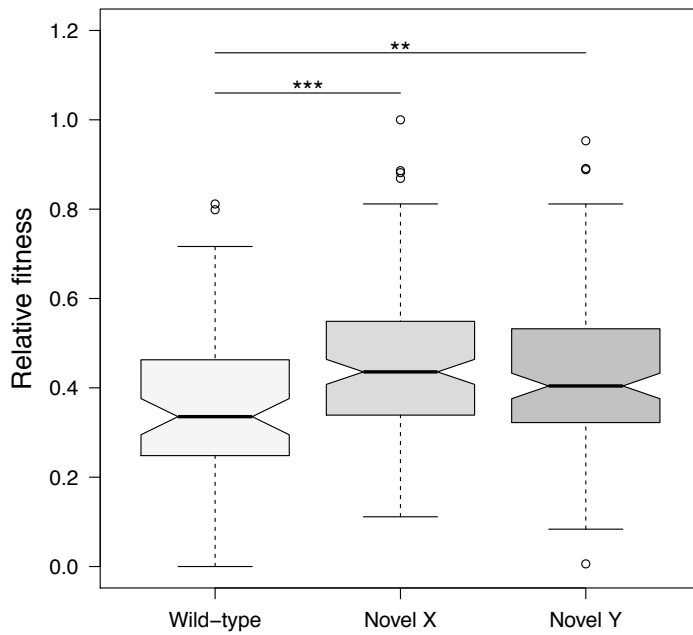


Figure 4.4: Relative male fitness for wild-type and novel genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.001$ ‘***’, $P < 0.01$ ‘**’).

Novel XY

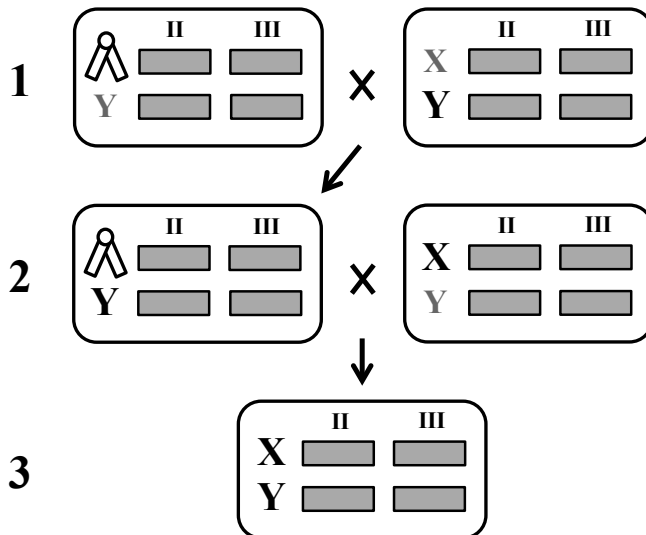


Figure 4.5: Protocol for crosses to create novel sex chromosome genotypes. Sex chromosome are denoted by letters (X/Y) and autosome II and III are depicted as rectangles. The double-X-chromosome is depicted as $\text{X} \text{X}$. Wild-type chromosome is either grey (population A) or black (population B). Only offspring used for the cross in the next generation are shown. For additional details see *Sex chromosome-Autosome interactions* in Methods.

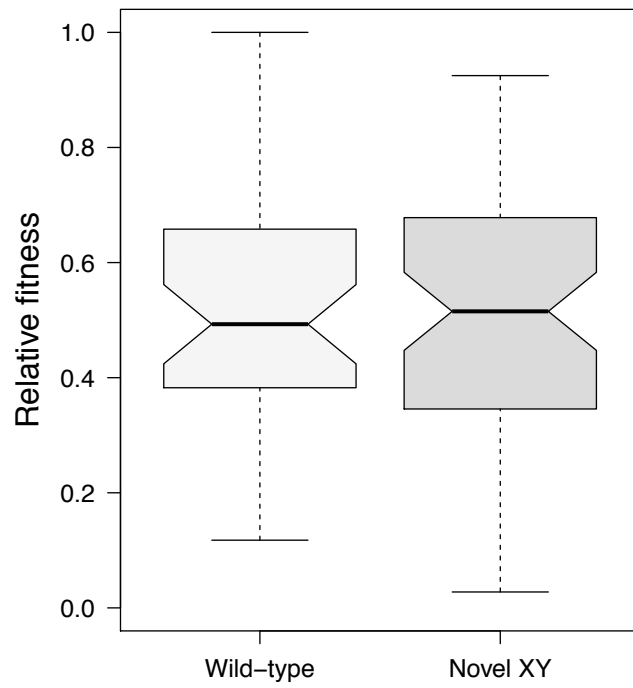


Figure 4.6: Relative male fitness for wild-type and novel sex chromosome genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles.

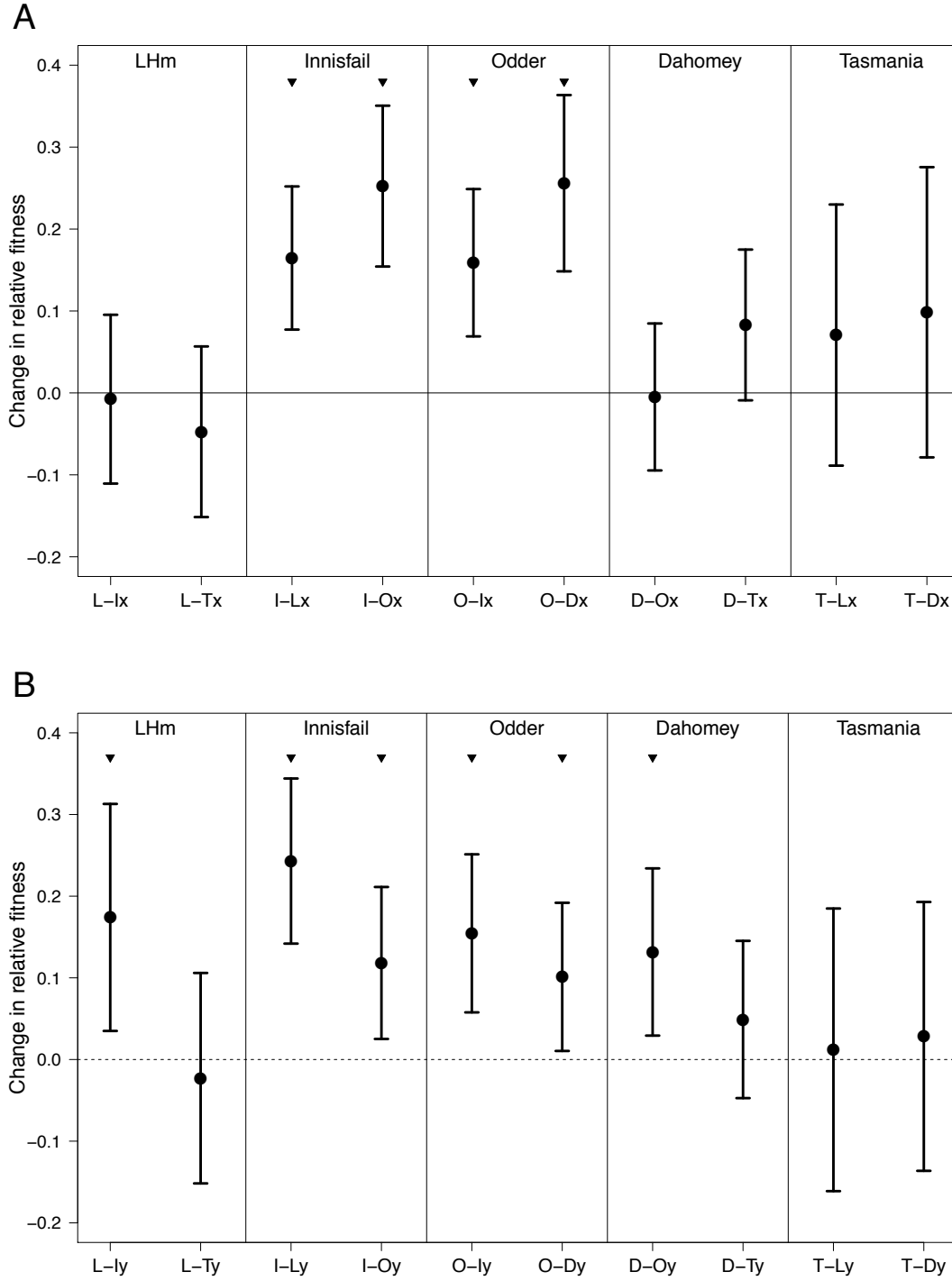


Figure 4.7: Change in relative fitness between novel genotypes and their wild-type populations. $\Delta\text{Fitness} = \omega_{\text{novel genotype}} - \omega_{\text{wild-type}}$ with bars indicating 95 % confidence intervals, calculated by bootstrap. A) Change in relative fitness for novel X genotypes. Each panel show which wild-types are subtracted from the novel X genotypes as plotted on the X-axis. The change in relative fitness is significantly different from 0 for four of the novel X genotypes (I-L_X, I-O_X, O-I_X, and O-D_X) marked by an arrow. B) Change in relative fitness for novel Y genotypes. Each panel shows which wild-types are subtracted from the novel Y genotypes as plotted on the X-axis. The change in relative fitness is significantly different from 0 for six of the novel Y genotypes (L-I_Y, I-L_Y, I-O_Y, O-I_Y, O-D_Y, and D-O_Y) marked by an arrow. The same annotation as in **Figure 4.1** is used for the novel genotypes. D: Dahomey, I: Innisfail, L: LH_M, O: Odder, and T: Tasmania.

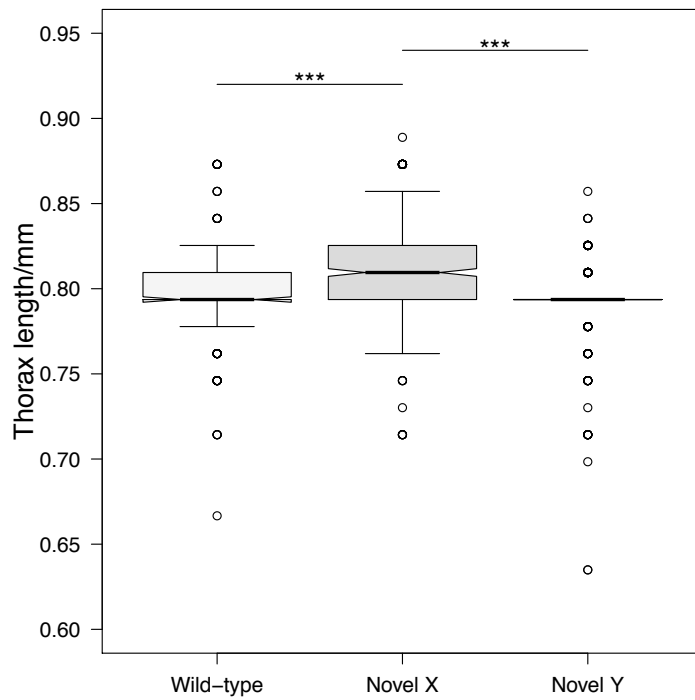


Figure 4.8: Thorax length in mm for wild-type and novel genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.001$ ‘***’).

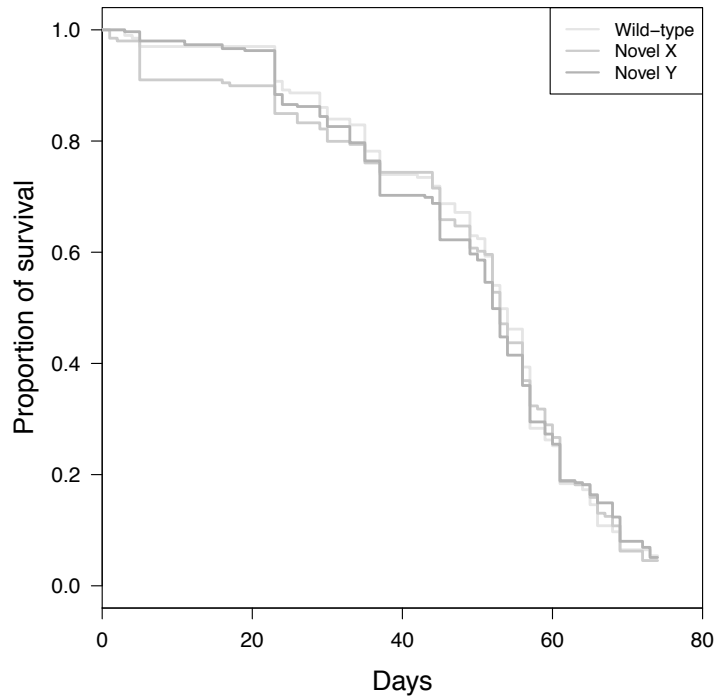


Figure 4.9: Male longevity in wild-type and novel genotype treatments. The proportion of survival is estimated from the model survfit in the R package Survival Analysis. Light grey line: wild-type, medium grey line: novel X, and dark grey line: novel Y treatment.

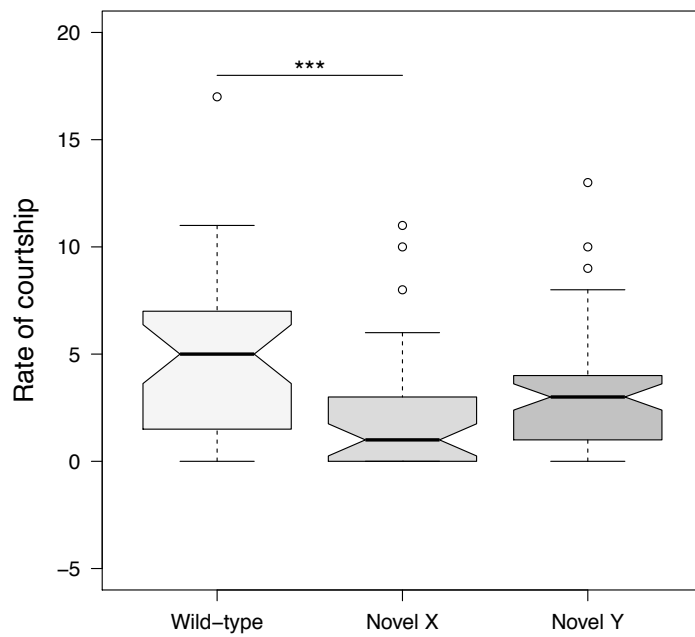


Figure 4.10: Rate of courtship for wild-type and novel genotype treatments. The rate of courtship is the total number of courtships over ten observations. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.001$ ‘***’).

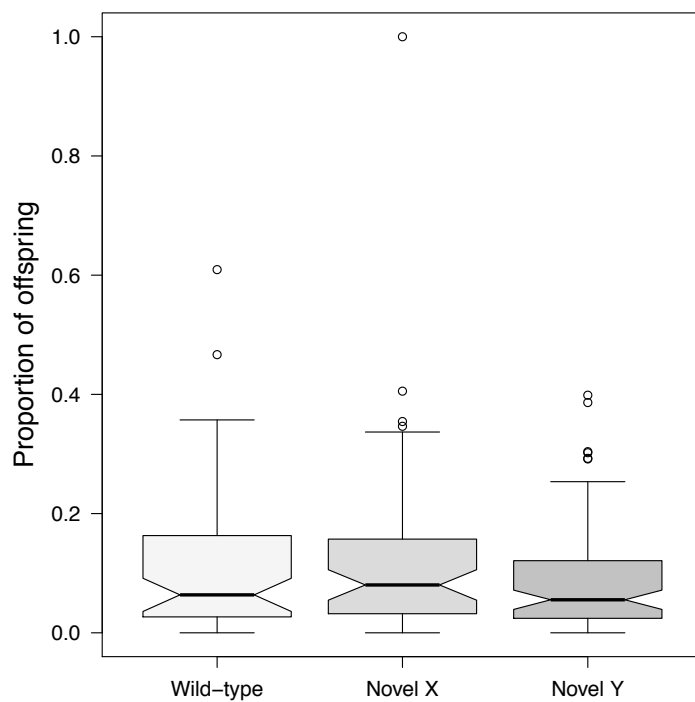


Figure 4.11: Sperm defence (P1) the proportion of offspring sired by target genotypes. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers.

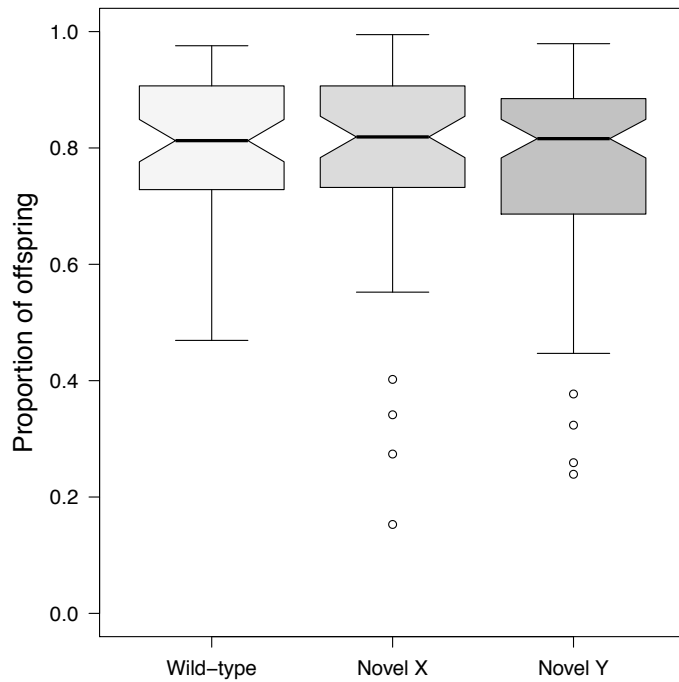


Figure 4.12: Sperm offence (P2) the proportion of offspring sired by target genotypes. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers.

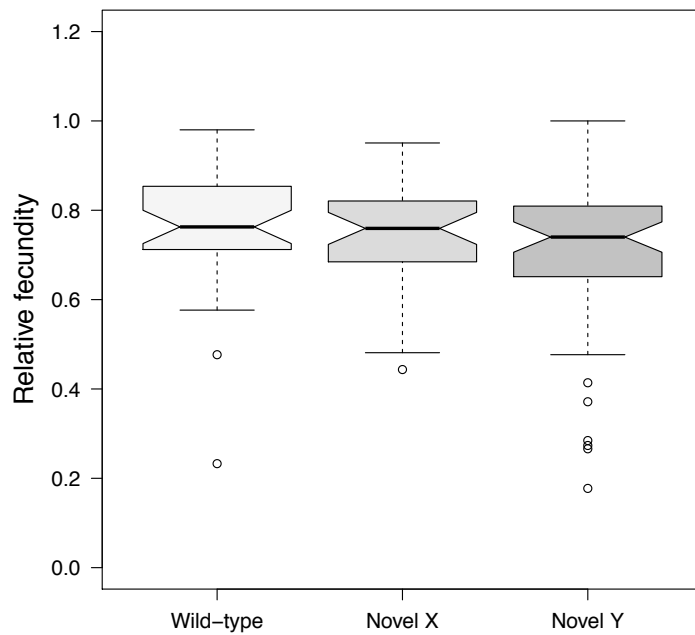


Figure 4.13: Male effect on relative female fecundity for wild-type and novel genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers.

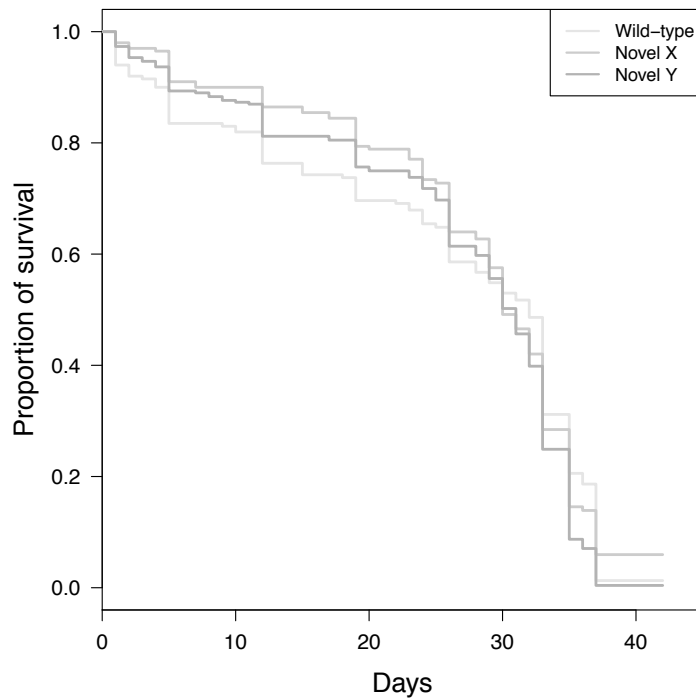


Figure 4.14: Female longevity with male harassment for wild-type and novel genotype treatments. The proportion of survival is estimated from the model `survfit` in the R package `Survival Analysis`. Light grey line: wild-type, medium grey line: novel X, and dark grey line: novel Y treatment.

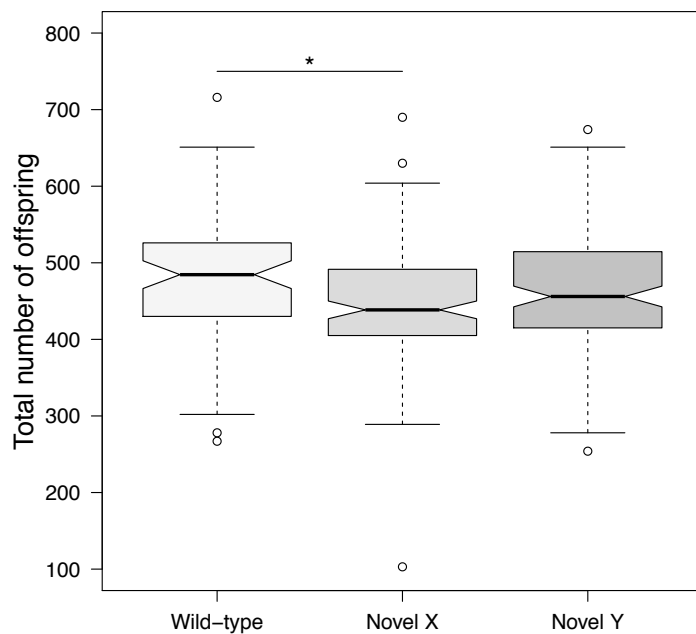


Figure 4.15: Total number of offspring from the male reproductive fitness assay. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.05$ ‘*’, $P < 0.01$ ‘**’).

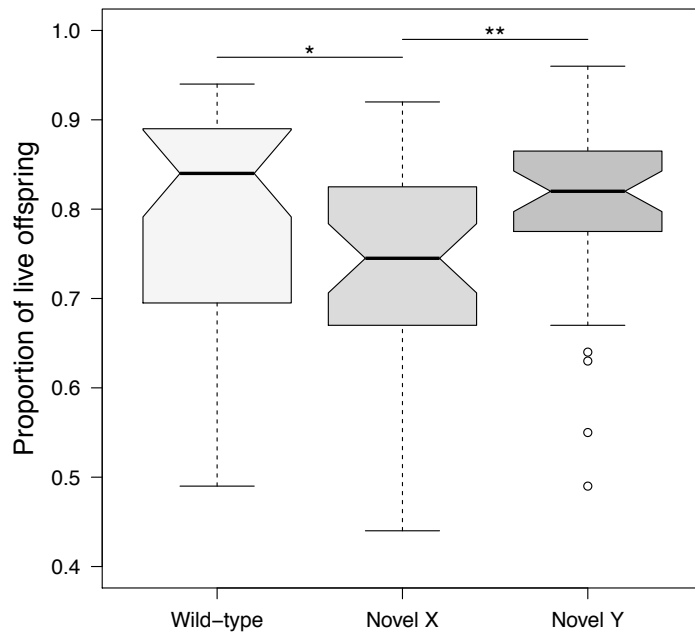


Figure 4.16: Egg-to-adult viability for wild-type and novel genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. Genotypes linked by a horizontal line indicate a significant difference (Tukey HSD; $P < 0.05$ (*), $P < 0.01$ (**)).

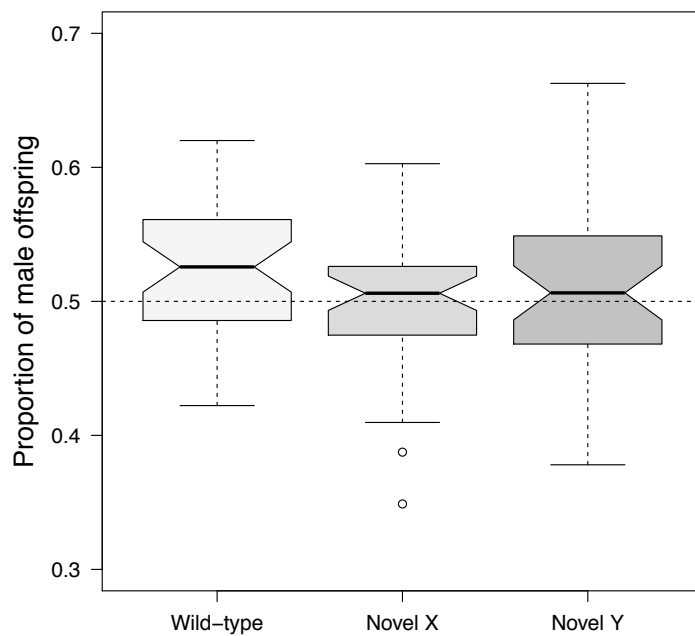


Figure 4.17: The proportion of male offspring for wild-type and novel genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. The dotted line indicates the expected equal sex ratio of 0.5.

Table 4.1: Results of ANOVA analysis of Generalized Linear Models and Linear Mixed-Effects Models with block as random factors.

Source	<i>df</i>	F	χ^2	<i>P</i>
Male fitness				
Population	24		98.06	$6.45e^{-11}$
Male fitness				
Genotype	2		16.78	0.0002
Male fitness, sex chromosome-autosome interactions				
Genotype	1	0.14		0.71
Thorax size				
Genotype	2		0.04	$3.55e^{-09}$
Male longevity				
Genotype	2		0.1	0.96
Courtship rate				
Genotype	2		53.61	0.0001
Sperm defence, P1				
Genotype	2		2.89	0.24
Sperm offence, P2				
Genotype	2		1.72	0.42
Male effect on female fecundity				
Genotype	2	1.75		0.18
Female longevity with male harassment				
Genotype	2		4.4	0.11
Total number of offspring				
Genotype	2		8.18	0.02
Egg-to-adult viability				
Genotype	2		10.85	0.004
Sex ratio				
Genotype	2		3.16	0.21

Table 4.2: The change in relative fitness with confidence intervals (CI) calculated by bootstrap for each of the novel genotype populations. The same annotation as in **Figure 4.1** is used for the novel genotypes. D: Dahomey, I: Innisfail, L: LH_M, O: Odder, and T: Tasmania.

	Δ Fitness	95 % CI
<hr/> Novel X		
L-I _X	-0.05	-0.11 – 0.10
L-T _X	-0.05	-0.15 – 0.06
I-L _X	0.16	<i>0.08 – 0.25</i>
I-O _X	0.27	<i>0.16 – 0.35</i>
O-I _X	0.15	<i>0.07 – 0.25</i>
O-D _X	0.27	<i>0.15 – 0.36</i>
D-O _X	-0.01	-0.10 – 0.08
D-T _X	0.04	-0.01 – 0.17
T-L _X	0.07	-0.09 – 0.23
T-D _X	0.10	-0.08 – 0.28
<hr/> Novel Y		
L-I _Y	0.14	<i>0.04 – 0.31</i>
L-T _Y	-0.04	-0.15 – 0.11
I-L _Y	0.22	<i>0.14 – 0.34</i>
I-O _Y	0.11	<i>0.03 – 0.21</i>
O-I _Y	0.14	<i>0.06 – 0.25</i>
O-D _Y	0.10	<i>0.01 – 0.19</i>
D-O _Y	0.10	<i>0.03 – 0.23</i>
D-T _Y	0.02	-0.05 – 0.14
T-L _Y	0.004	-0.16 – 0.19
T-D _Y	0.02	-0.14 – 0.20

Chapter 5: Experimental evolution of novel pairs of sex chromosomes in *Drosophila melanogaster*

Abstract

Results from previous chapter indicate that the sex chromosomes in *Drosophila melanogaster* might participate in a perpetual cycle of antagonistic coevolution. To follow-up on these results, I designed an evolution experiment where the novel pairs of sex chromosomes were allowed time to co-adapt. Whereas the first experiment examined the start of a cycle of antagonistic coevolution, this experiment examined how the cycle might proceed. After 25 generations of experimental evolution, the initial increase in relative male fitness disappeared, and there were no longer any significant differences between novel genotypes and their wild-type counterparts. These results add further support to the idea that a cycle of antagonistic coevolution can occur between sex chromosomes, and illustrates how each independent cycle can increase genetic and phenotypic divergence between populations of *D. melanogaster*.

Introduction

In species with two sexes, conflict can impact the shared genome when the evolutionary interests (e.g. mating strategies, parental investment) of each sex are in opposition to each other. Sexual conflict is an evolutionary dynamic that can prevent each sex from reaching individual fitness optima, and can be particularly harmful when alleles beneficial to one sex are simultaneously disadvantageous to the other, e.g. sexual antagonism (Parker, 1979; Bonduriansky and Chenoweth, 2009). The antagonistic interactions between alleles means that this conflict has been likened to the ‘Red Queen Hypothesis’ of antagonistic coevolution of species (Van Valen, 1973), where one species can increase fitness through harmful interaction with another species, and so incites a counter-adaptation in the second species to reduce the harmful interaction, which then starts a cycle of antagonistic coevolution between the two species (Rice and Holland, 1997; Brockhurst et al., 2014). Rice and Chippindale (2002) presented a hypothesis for antagonistic coevolution between sexually antagonistic variation in the genome and sex-limited genes. They theorised that a change in sexually antagonistic variance in the direction of either sex (i.e. an accumulation of either male or female beneficial sexual antagonistic alleles) would

push the other sex further away from its optimum fitness value (i.e. increased gender-load). As each sex has a pool of genes with sex-limited expression, these could be used to ameliorate the increased gender-load and thereby counteract the negative effects of the sexually antagonistic genes, and so led to antagonistic coevolution (Rice and Chippindale, 2002). I extrapolated this hypothesis to suggest a model for antagonistic coevolution between the sex chromosomes in *D. melanogaster* (Chapter 4).

Sex chromosomes are a unique part of the genome because of their sex-limited mode of inheritance, their specialised sex-limited gene expression, and sex determination (Mank, 2012). Because of these special features, and the inherent conflict between the two sexes, it follows that conflict could occur between the sex chromosomes themselves, potentially resulting in antagonistic coevolution. Like the ‘Red Queen Hypothesis’, the cycle of antagonistic coevolution would start with an adaption beneficial to one sex. Any changes in sexually antagonistic variance that benefits one sex will lead to a decreased in fitness for the second sex, increasing gender-load. Therefore, a Y-chromosome (chrY) encoded adaptation that alters sexually antagonistic variance to benefit male fitness will result in decreased in female fitness. It has been shown that the chrY can affect the rest of the genome through epigenetic interactions, altering expression of non Y-linked genes (Lemos et al., 2008). Selection through female-limited gene expression on the X-chromosome (chrX) can counter the negative effects, and effectively restoring female fitness. As the compensation by the chrX offsets the benefits to the chrY, male fitness will decrease again. This cycle of antagonistic coevolution may then be restarted by a new adaptation of the chrY that increases male fitness.

I have previously shown that by decoupling the sex chromosomes from their ancestral pairings it was possible to disrupt the cycle of coevolution between the sex chromosomes to benefit of males. I found a significant increase in male reproductive fitness at the cost reduced egg-to-adult viability (Chapter 4). The other half of the cycle of coevolution is the counter-adaptation of females; these counter-adaptations ameliorate the negative fitness effect of the chrY, restoring female fitness. This increased female fitness causes a reduction in male benefits, resulting in a relative decrease in male fitness. To complete the empirical test of *D. melanogaster* sex chromosome coevolution, I performed an evolution experiment on the novel genotypes I had created by exchanging sex chromosomes between five wild-type

populations (Chapter 4). If the sex chromosomes coevolve and this interaction is antagonistic, I hypothesized that after 25 generations of counter-adaptation between the novel pairs of sex chromosomes should remove any negative effects on female fitness; this should be demonstrable by a decrease in male fitness back to the wild-type levels. I re-measured relative male fitness after 25 generations of novel sex chromosome pair co-adaptation, to investigate whether the gain in male fitness observed at generation 0 is lost by generation 25.

Methods

Stocks

I selected the four novel X and four novel Y populations with the greatest divergence in male fitness from their wild-type counterpart (see Chapter 4 and Table 5.1), to investigate if novel pairs of sex chromosomes co-adapt during an evolution experiment.

Stocks were maintained using the standard LH_M culturing protocol, at 25°C with 60% relative humidity, on a 12-12 light-dark cycle, and fed on cornmeal-molasses-yeast medium (Rice et al., 2005). Flies were kept on a two-week generation time with non-overlapping generations. On day 12 after oviposition, 16 pairs of adult flies were randomly selected from the pool of adults and placed in a food-vial with 6 mg yeast. The pairs were left in the vials for two days, and then flipped into fresh vials without yeast. The females were allowed to oviposit for 18 hours, after which all adult flies were discarded. The number of eggs was reduced to 150-200 per vial to maintain constant larval density.

Evolution experiment protocol

The three genotype treatments (novel X, novel Y, and wild-type) were kept in an adult breeding population of 160 pairs (♀ 16:16 ♂ per vial) in 10 vials, with a minimum of 1,500 offspring. There were four populations in each genotype group, with each population kept in two replicates.

- i. *Novel X genotypes* – Males carrying a novel were crossed at each generation to females who carried a double-bound chrX (DX) and a chrY (Figure 5.1, Novel X). Because only half of the offspring of DX females survive to

adulthood, the number of eggs were reduced to ~300 for these genotypes. The other half are inviable because they receive the wrong complement of sex chromosomes, either two chrY or three chrXs.

- ii. *Novel Y genotypes* – Each generation males carrying a novel chrY were crossed to X/X females (Figure 5.1, Novel Y).
- iii. *Wild type populations* – The four wild type populations were kept at the same population size as the novel genotypes.

Male fitness assay

Male fitness was measured as the proportion of live offspring sired by the target genotype males. Five adult target males (day 12 after oviposition) were combined with ten competitor males and fifteen virgin females in a yeasted vial. The competitor flies are from the outbred LH_M population homozygous for the visible brown eye (*bw*) genetic marker (LH_M-*bw*), which is recessive to the wild-type red eye-colour allele. After two days, 14 females were isolated in single test tubes with food medium and allowed to oviposit for 18 hours. Females were then discarded, the test tubes were kept under standard LH_M conditions for 12 days, and the adult offspring from each tube were counted and scored for eye-colour to assign paternity to either target or competitor males. Since the wild-type red-eye colour allele is dominant to the *bw* allele, all red-eyed offspring can be assigned to the target males. This assay was performed in two blocks with three experimental replicates per replicate population in each, providing fitness estimates based on data from 60 individuals per genotype. Relative fitness was calculated by dividing the fitness for each replicate by the maximum fitness across all replicates.

Statistical procedures

All the statistical analyses were conducted in R version 3.2.3 (R Core Team, 2015). I used the Linear Mixed-Effects Model (*lmer*) command from the R package **lme4** (Bates et al., 2015) with experimental block and replicate populations as random factors. I fitted two separate models for male fitness with either population or genotype as a fixed factor due to collinearity between these two factors. Significance tests were obtained using the *anova* command in R. To estimate the change in relative fitness between evolved novel genotypes and their wild type background, I calculated: $\Delta\text{Fitness} = \omega_{\text{novel genotype}} - \omega_{\text{wild type}}$. The 95% confidence intervals around $\Delta\text{Fitness}$ was

calculated by bootstrap, randomly resampling 11 out of 12 data points and recalculating Δ Fitness 10,000 times.

Results

After 25 generations of evolution experiment, I performed a male fitness assay in order to test the extent of chrX counter-adaptation to antagonistic chrY effects I found no significant effect of genotype on male fitness ($P = 0.68$, Figure 5.2 and Table 5.2), nor any significant difference between the genotype treatments ($P = 0.84$, Figure 5.3 and Table 5.2).

The confidence intervals for Δ Fitness were not significantly different from 0 after 25 generations, except for one novel X genotype, I-O_X (Figure 5.4 and Table 5.3). When comparing Δ Fitness between the start and the end of the evolution experiment, all 95% confidence intervals are overlapping and therefore not significantly different from each other (Figure 5.5 and Table 5.3). However, there is a clear trend that Δ Fitness between the evolved genotypes and their wild-type counterparts diminish after 25 generations.

Discussion

I wanted to empirically test if the sex chromosomes in *D. melanogaster* coevolve and whether this coevolution is antagonistic. Previously, I disrupted coevolution between pairs of sex chromosomes in five wild-type populations by introducing a novel sex chromosome (Chapter 4). I found that decoupling the interaction between the sex chromosomes led to an increase in male relative fitness for 10 out of 20 novel genotypes. I found indications that the coevolution was antagonistic; the increase in male fitness came at a cost, observable as a decrease in offspring egg-to-adult viability.

To further investigate whether coevolution between sex chromosomes is antagonistic, I hypothesised that the chrX should counter a gain in male fitness by ameliorating the negative effect of the chrY restoring female fitness again while simultaneously decreasing male fitness. Of the 10 novel genotypes that showed significantly higher fitness than their wild-type counterparts, I chose eight complimentary novel genotypes (four novel X and four novel Y) to investigate whether the chrX could ameliorate negative effects of the chrY. I performed an

evolution experiment to allow novel pairs of sex chromosomes time to coevolve with each other, and found that after 25 generations novel genotypes no longer had a fitness advantage over their wild-type counterparts. Indeed, I found that after 25 generations the change in fitness ($\Delta\text{Fitness}$) between novel genotypes and their wild-type counterparts was no longer significantly different from 0, except for one genotype, I-O_X. For all genotypes, $\Delta\text{Fitness}$ had diminished in magnitude after 25 generations, indicating an improvement of the negative effects after disrupting coevolution between sex chromosomes at the start of the evolution experiment, which could indicate a strong selective pressure to return the sex chromosomes to their original state.

A weakness with this experimental setup is the use of DX females for crosses in the novel X genotypes. This alters the normal pattern of inheritance for the sex chromosomes, such that sons inherit chrYs from their DX mothers and chrXs from their fathers. This is problematic, as I hypothesized that a response to decreased female fitness would be driven by the actions selecting on the female-limited genes on the chrX; in this case the chrX is not present in females in the novel X genotypes. This experimental setup is similar to a male-limited chrX experiment done by Abbott *et al.* (2013), where they found that limiting selection on the chrX to males resulted in increased male fitness. Using the same setup, my novel X genotypes might increase in fitness rather than decrease as expected. However, I did find a decrease in male relative fitness, so it seems that disrupting coevolution between sex chromosomes evoked a stronger selection pressure than the male-limited inheritance of the chrX. While I found no effect of interaction with the autosomes in the previous experiment (Chapter 4), I cannot exclude that the counter-adaptation I observed in this experiment can be exclusively attributed to the chrX. Indeed, the results from novel X genotypes imply that the autosomes may also be involved in this counter-adaptation. To account for the problems with the experimental setup, I repeated the evolution experiment without using DX females in novel X genotypes. Rather, I created females with two novel chrXs, so that all chrXs in both males and females originated from the same populations, and were from a different population than the rest of the genome. However, this new evolution experiment is still ongoing, so I cannot conclude whether this altered experimental setup affects the results of the experimental evolution or not.

To complete the empirical test of coevolution between the sex chromosomes in *D. melanogaster*, I first disrupted the coevolution by introducing a novel sex chromosome and then allowed time for the novel pairs to co-adapt during an evolution experiment. If the coevolution between the sex chromosomes was antagonistic, disruption of the interaction would be harmful to one sex but beneficial to the other, which I observed in Chapter 4. This perturbed state should then settle in time and the novel genotypes should return to their original state, which is what I observed in this experiment. Taken together with the results from Chapter 4, this indicates that sex chromosomes do coevolve in an antagonistic manner. Accordingly, this novel insight into the interactions between sex chromosomes could help further our knowledge about genetic and phenotypic diversity at the population level in a species.

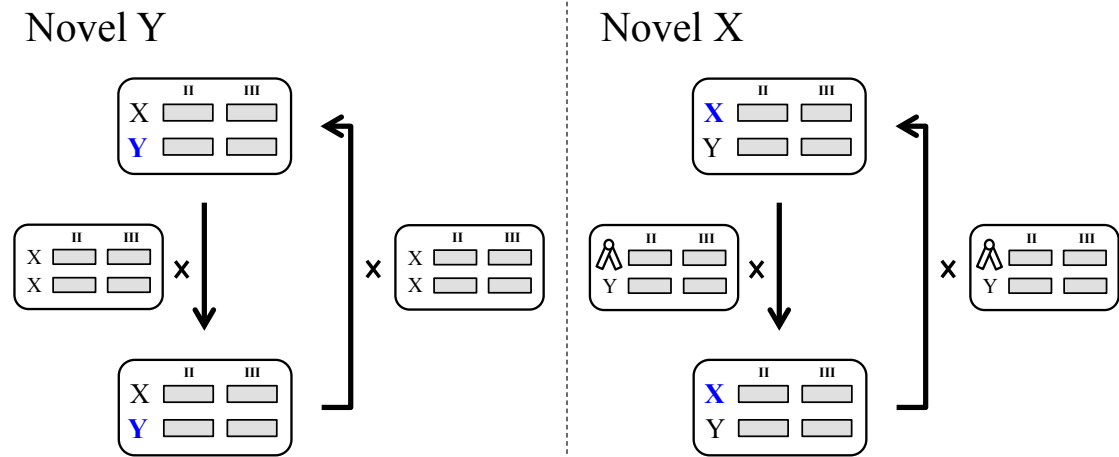


Figure 5.1: Protocol for the experimental evolution of novel X/Y chromosomes. Sex chromosomes are denoted by letters (X/Y) and autosome II and III are depicted as light grey rectangles. The double-X-chromosome is depicted as Δ . For additional details see *Evolution experimental protocol* in Methods.

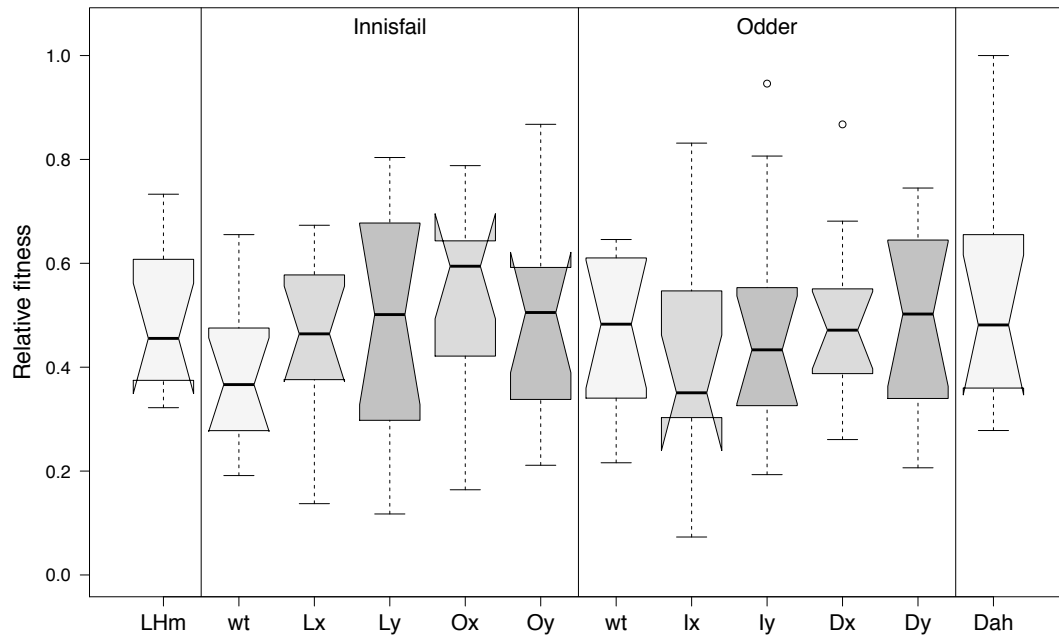


Figure 5.2: Relative male fitness for the eight evolved genotypes and the four wild-type populations. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers. The wild-type genotypes are plotted as reference for the four evolving genotypes in the two middle panels. The capital letter indicates which population the sex chromosomes originate from and sex chromosomes are denoted by x or y. D(ah): Dahomey, I: Innisfail, L: LH_M, and O: Odder.

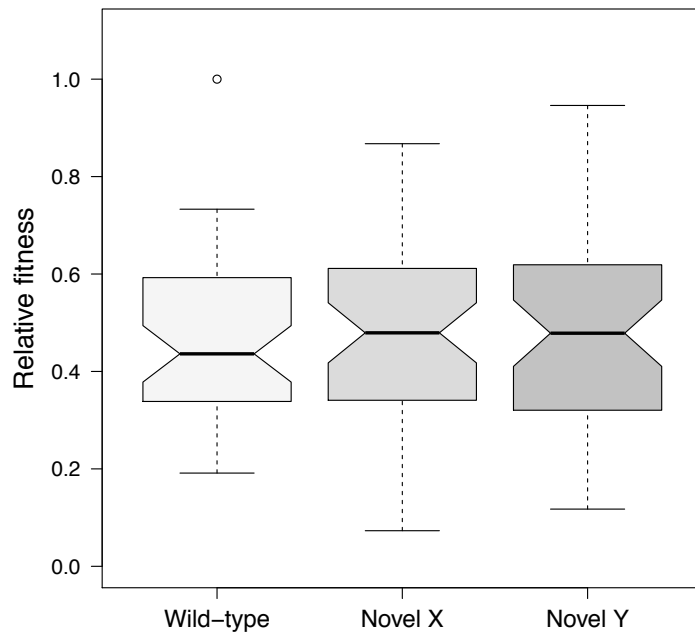


Figure 5.3: Relative male fitness for wild-type and evolved genotype treatments. Horizontal lines indicate the median, with notches corresponding to the 95 % confidence interval ($\pm 1.58 \times \text{IQR}/\sqrt{n}$). The boxes show the interquartile range (IQR) and the whisker the 5th and 95th percentiles. Circles indicate outliers.

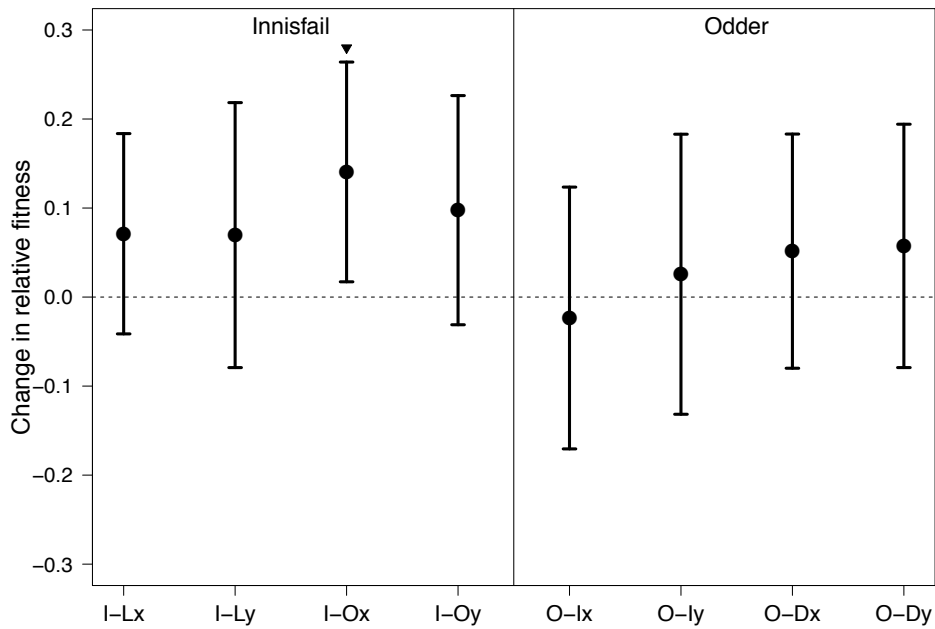


Figure 5.4: Change in relative fitness between novel genotypes and their wild-type populations. $\Delta\text{Fitness} = \omega_{\text{novel genotype}} - \omega_{\text{wild type}}$ with bars indicating 95 % confidence intervals, calculated by bootstrap. Each panel shows which wild-types the novel genotypes are subtracted from. An arrow indicates $\Delta\text{Fitness}$ that are significantly different from 0. The capital letter indicates which population the sex chromosomes originate from and sex chromosomes are denoted by x or y. D: Dahomey, I: Innisfail, L: LH_M, and O: Odder.

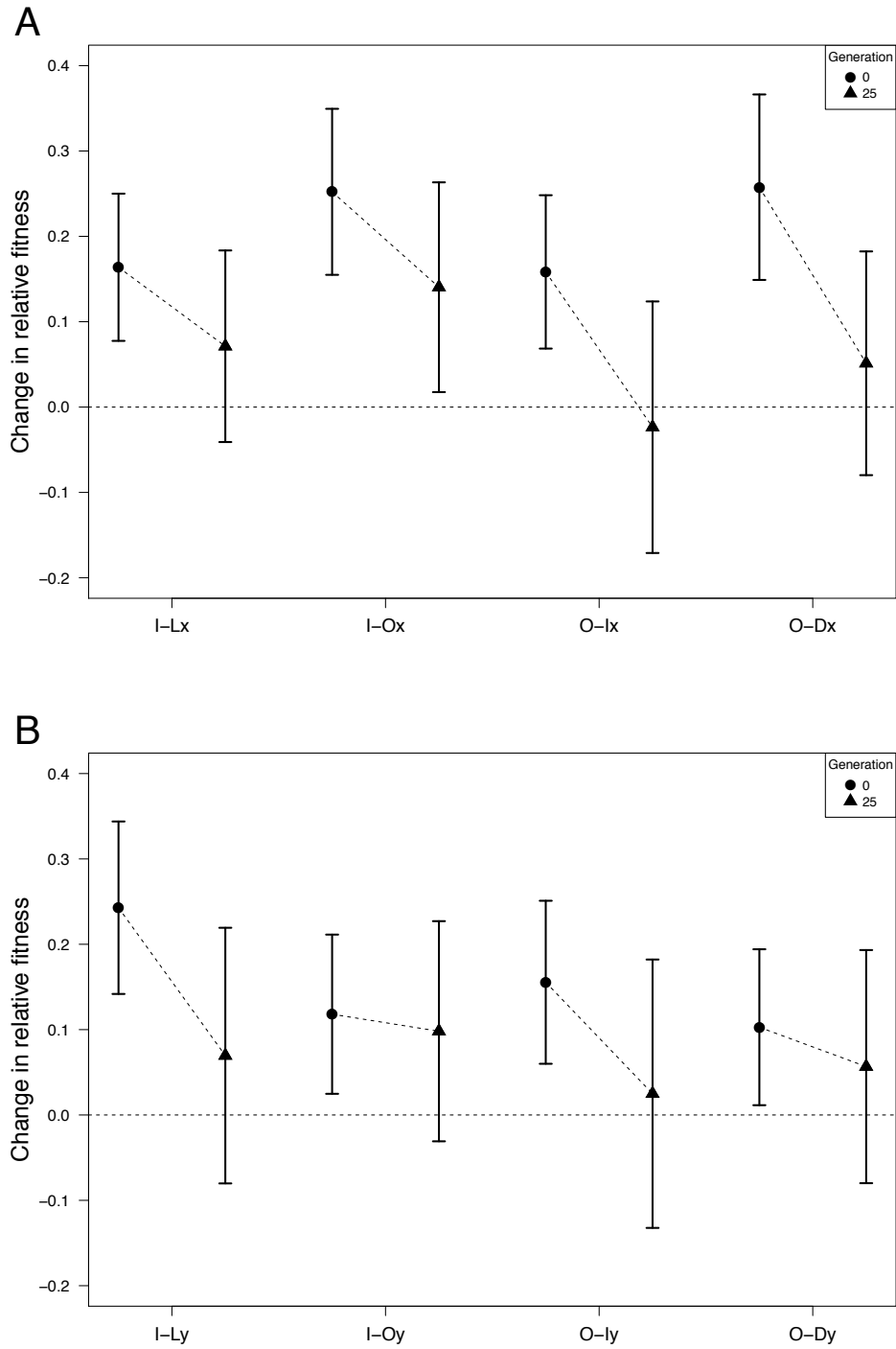


Figure 5.5: Comparison of Δ Fitness at the start and end of the evolution experiment. A: Novel X genotypes and B: Novel Y genotypes. The bars indicate 95 % confidence intervals, calculated by bootstrap. The dash line connects the same genotype at generation 0 (circle) and generation 25 (triangle). The capital letter indicates which population the sex chromosomes originate from and sex chromosomes are denoted by x or y. D: Dahomey, I: Innisfail, L: LH_M, and O: Odder.

Table 5.1: The eight genotypes chosen for the evolution experiment. The table shows which wild-types the sex chromosomes derive from and which wild-types provided the rest of the genome.

<u>Novel X</u>		
Name	Chromosome X	Genetic background
I-L _X	LH _M	Innisfail
I-O _X	Odder	Innisfail
O-D _X	Dahomey	Odder
O-I _X	Innisfail	Odder

<u>Novel Y</u>		
Name	Chromosome Y	Genetic background
I-L _Y	LH _M	Innisfail
I-O _Y	Odder	Innisfail
O-D _Y	Dahomey	Odder
O-I _Y	Innisfail	Odder

Table 5.2: Results of ANOVA analysis of linier mixed model with block as a random factor

Source	<i>df</i>	χ^2	<i>P</i>
Male fitness			
Population	11	8.39	0.68
Male fitness			
Genotype	2	0.34	0.84

Table 5.3: The change in relative fitness between the novel evolved genotypes and the wild-type background at the start (generation 0) and end (generation 25) of the evolution experiment. Confidence intervals (CI) are calculated by bootstrap. The same annotation is used for the novel genotype as **Table 5.1**.

	Generation	Δ Fitness	95 % CI
Novel X			
I-L _X	0	0.16	<i>0.07 – 0.25</i>
	25	0.07	-0.04 – 0.18
I-O _X	0	0.25	<i>0.15 – 0.35</i>
	25	0.14	<i>0.02 – 0.26</i>
O-I _X	0	0.16	<i>0.07 – 0.25</i>
	25	-0.02	-0.17 – 0.12
O-D _X	0	0.26	<i>0.15 – 0.36</i>
	25	0.05	-0.08 – 0.18
Novel Y			
I-L _Y	0	0.24	<i>0.14 – 0.34</i>
	25	0.07	-0.08 – 0.22
I-O _Y	0	0.12	<i>0.02 – 0.21</i>
	25	0.10	-0.03 – 0.23
O-I _Y	0	0.15	<i>0.06 – 0.25</i>
	25	0.03	-0.13 – 0.18
O-D _Y	0	0.10	<i>0.01 – 0.19</i>
	25	0.06	-0.08 – 0.19

Chapter 6: General discussion

Sexual conflict can limit adaptation by displacing both sexes from their fitness optima. As inter- and intralocus sexual conflict impacts the interactions between the genomes of the two sexes, understanding these mechanisms can help illuminate which evolutionary forces shape the genome, and how these forces can affect complex phenotypic traits. The aim of this thesis was to empirically test which phenotypic and genetic traits that experience inter- and intra-locus sexual conflict. I added to the list of phenotypic traits that have been shown to be sexually antagonistic (**Chapter 2**) and showed the effect of female-limited X-chromosome (FLX) evolution on gene expression (**Chapter 3**). Furthermore, I examined how antagonistic coevolution of the sex chromosomes can help shape the evolution of the genome (**Chapters 4 and 5**).

Interlocus sexual conflict

Antagonistic coevolution

Rice and Holland (1997) proposed a process by which interlocus sexual conflict could lead to antagonistic coevolution between the two sexes. They suggested that this *interlocus contest evolution* would have the greatest effect in sex-limited genes. Since chromosome Y (chrY) is sex-limited and there is an imbalance in the occurrence of chromosome X (chrX) in the two sexes, I expanded their theory to specifically include coevolution between the two sex chromosomes (**Chapter 4**).

Through a round-robin population cross I found that exchanging either chrX or chrY had significant effects on male reproductive fitness. 10 out of 20 novel genotypes had significantly increased fitness compared to their corresponding wild-type populations. These results indicate that disrupting the interactions between chrX and chrY can have significant effects on male fitness. In contrast, I found no evidence that the autosomes were involved in these effects on male fitness. I further examined which phenotypic traits were involved in increased male fitness. Many phenotypic traits, such as courtship rate (Partridge et al., 1987a), large body size (Partridge et al., 1987; Pitnick, 1991), and sperm competition (Clark et al., 1999), have been shown to increase male fitness. I only found evidence that increased body size were a trait through which novel male genotypes could increase their fitness.

Since coevolution between the sexes is often predicted to be antagonistic (Rice and Holland, 1997; Perry and Rowe, 2015), I wanted to examine any potentially antagonistic effects of the increased male fitness. I therefore examined traits that might contribute to a reduction in female fitness, such as reduced female lifespan after mating, and increased rates of egg-laying. I also looked at male lifespan as it has been shown to be negatively affected by mating (Prowse and Partridge, 1997). However, I detected no change to these traits in the novel genotypes. Instead, I found evidence of effects on offspring viability. This indicates that the antagonistic interaction between the two sexes is postzygotic and indirect.

Since this experiment was performed by exchanging one sex chromosome only, the effects shown here might not be apparent in a standard population cross, where half of the genome is exchanged. Thus, this experimental design enabled me to observe coevolution between sex chromosomes in isolation.

To complete the investigation, and test if the interactions between the sex chromosomes are coevolutionary and antagonistic, I performed an evolution experiment on the novel pairs of sex chromosomes from Chapter 4 (**Chapter 5**). The rationale being that if the novel pairs of sex chromosomes had disrupted the coevolutionary balance between the sexes, allowing populations with these novel pairings to evolve may re-establish this balance, and the consequent effects on male fitness would disappear.

After 25 generations of experimental evolution there were no significant differences between the male fitness of novel genotypes and their corresponding wild-type populations. This indicates that the novel pairs of sex chromosomes had indeed coadapted to each other by the end of the experiment. The fact that sex chromosomes coadapted so quickly indicates that disrupting the interaction between them was rapidly selected against.

The results from these two complementary experiments provide empirical evidence that one cycle of antagonistic coevolution can occur between the sex chromosomes in *D. melanogaster*, and that the effects of such antagonistic coevolution are both pre- and postzygotic. Despite the cosmopolitan distribution of *D. melanogaster*, sexual isolation has been reported between populations in Zimbabwe and populations from other continents (Wu et al., 1995), and candidate genes for mate choice in Zimbabwean females were disproportionately X-linked (Bailey et al., 2011).

So, it is possible that antagonistic coevolution between the sex chromosomes could be associated with early speciation events.

Intralocus sexual conflict

Sexually antagonistic phenotypic traits

There is growing empirical evidence for sexually antagonistic phenotypic traits in a number of species. These traits include reproductive fitness [Collared flycatcher (Brommer et al., 2007), *Drosophila melanogaster* (Prasad et al., 2007; Abbott et al., 2010), ground cricket (Fedorka and Mousseau, 2004), and Red deer (Foerster et al., 2007)], body size [Collared flycatcher (Merilä et al., 1997; Merila et al., 1998) and *D. melanogaster* (Prasad et al., 2007; Abbott et al., 2010)], development time and developmental stability [*D. melanogaster* (Prasad et al., 2007; Abbott et al., 2010)], and locomotory activity [*D. melanogaster* (Long and Rice, 2007)]. To understand the constraints that sexually antagonistic selection imposes on natural selection, it is useful to study these traits in detail and investigate if sexually antagonistic selection is equally strong in both sexes (**Chapter 2**).

As most sex-limited evolution experiments before had employed male-limited selection (Rice, 1996, 1998; Prasad et al., 2007; Abbott et al., 2013), I carried out a complementary female-limited X-chromosome evolution experiment, which to my knowledge is the first time this has been attempted. By limiting selection of the chrX to females, I showed that body size moved towards an assumed female optimum in both females and males. Traits such as reproductive fitness did not produce similarly clear results, while some, such as growth rate and locomotion activity, produced results that were partly contradictory to previous studies (Prasad et al., 2000; Long and Rice, 2007). I found an increase in dark pigmentation on the female abdomen, which was not predicted to change under selection, and could be a correlated response to the FLX selection or to the chrX balancer (First Multiple; FM).

These results add to the growing list of empirical evidence that intralocus sexual conflict does exist for a number of phenotypic traits related to fitness and body size. This evolution experiment was aimed to investigate the magnitude of response to female-limited X-chromosome selection of those phenotypic traits that had previously been found to respond to male-limited selection (Prasad et al., 2007; Abbott et al., 2010). I did not detect an equal opposite response, which could be because chrX is

already close to the female optimum, because the chrX it spends two-thirds of its time in females, or because of the confounding effects of the FM balancer. The control treatment for the FM balancer showed evidence of long-term adaptation to the balancer, leading to increased fitness when the genome was expressed without the balancer. This makes interpreting the results of the FLX evolution experiment more difficult, as it can be hard to separate the effects of the selection from the effects of the balancer. Since the FM balancer does not recombine, it would be expected that it would accumulate deleterious mutations over time due to genetic hitchhiking (Futuyma, 2009), which would have a negative effect on the fitness of the flies. A newly created balancer chromosome could therefore have a different impact on flies than an old one. Many different chrX balancers are available (Ashburner et al., 2005), and it would be possible to design an experiment to compare the effects of each balancer, allowing identification of effects attributable to the individual balancers.

Gene expression

As it has become simpler and more economically viable to sequence both the genome and the transcriptome, there has been more focus on identifying the genetic architecture underlying sexually antagonistic phenotypic traits. I had a unique opportunity to investigate what effects the selection applied during the FLX evolution experiment had on the transcriptome of both females and males.

There was no clear pattern of differences in gene expression between the five female genotypes, as I found no clear grouping of replicated populations within each female genotype. I found an indication of ‘feminisation’ of gene expression in FLX males compared to Cwt but also in CFM males, which adds to my hypothesis that adaptation to the FM balancer did affect the genome.

There was no clear effect of the FLX evolution experiment on gene expression in either females or males, and it seems that the response to the FM balancer complicates interpretation of the results. For future analyses it would be worth examining which genes had changed through adaptation to the FM balancer.

Conclusion

In this thesis, I aimed to enhance the understanding of sexual conflict on a genomic level. I carried out two large experiments to demonstrate the occurrence of inter- and

intralocus sexual conflict in *D. melanogaster*, a comprehensive population cross, and an experimental evolution experiment.

In the first experiment, I investigated if the sex chromosomes in *D. melanogaster* coevolve. I found that disrupting the stability between sex chromosomes increased male fitness, but that this came at a cost to offspring viability. To test if this coevolution was antagonistic, I performed a follow-up evolution experiment to examine if the stability between the sex chromosomes could be restored, which I confirmed. Collectively, these experiments demonstrate that sex chromosomes coevolve in an antagonistic manner. It also supplements growing evidence that the Y-chromosome is not an inactive part of the genome, but may interact epistatically with the rest of the genome to the benefit of males.

To examine intralocus sexual conflict, I performed a female-limited X-chromosome evolution experiment. While some phenotypic traits moved towards presumed female optima, most traits did not vary between selected and wild-type flies. This could be because there was little pre-existing sexually antagonistic variation on chrX for selection to act upon, or because chrX was already close to the female optimum so selection in that direction would be weak.

Both of these studies add to the existing body of empirical data for sexual conflict, expanding on current knowledge of how sexual conflict influences the genome and what impact this could have on the evolvability of the two sexes.

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